

UNIVERSITY
OF TASMANIA



**INVESTIGATIONS OF SKELETAL ANOMALIES IN TRIPLOID
ATLANTIC SALMON (*Salmo salar* L. 1758) IN FRESHWATER WITH
PARTICULAR FOCUS ON LOWER JAW DEFORMITY (LJD)**

Gianluca Amoroso

Master of Evolutionary Biology and Ecology (Aquaculture)

Bachelor of Natural Sciences

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Institute for Marine and Antarctic Studies

University of Tasmania

Launceston, Tasmania

May 2016

DECLARATION OF ORIGINALITY

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

(signature)

22/05/2016

(date)

AUTHORITY OF ACCESS

This thesis may be made available for loan and limited copying and communication in accordance with the Copyright Act 1968.

(signature)

22/05/2016
(date)

STATEMENT REGARDING PUBLISHED WORK

The publishers of the papers comprising Chapters 2nd to 4th, inclusive, hold the copyright for that content, and access to the material should be sought from the respective journals. The remaining non published content of the thesis may be made available for loan and limited copying and communication in accordance with the above Statement of Access and the Copyright Act 1968.

Due to the inclusion of published material there is unavoidable repetition of material between chapters in this thesis.

(signature)

22/05/2016

(date)

CO-AUTHORSHIP

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

Gianluca Amoroso, Institute for Marine and Antarctic Studies, University of Tasmania

Chris G. Carter, Institute for Marine and Antarctic Studies, University of Tasmania

Jennifer M. Cobcroft, Institute for Marine and Antarctic Studies, University of Tasmania

Mark B. Adams, Institute for Marine and Antarctic Studies, University of Tasmania

Tomer Ventura, Genecology Research Centre, School of Science and Engineering, University of the Sunshine Coast

Abigail Elizur, Genecology Research Centre, School of Science and Engineering, University of the Sunshine Coast

(signature)

25/05/2016
(date)

Professor Chris Carter

Main Supervisor

Institute for Marine and Antarctic Studies

University of Tasmania, Hobart

(signature)

22/05/2016
(date)

Gianluca Amoroso

PhD candidate

Institute for Marine and Antarctic Studies

University of Tasmania, Hobart

STATEMENT OF ETHICAL CONDUCT

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

(signature)

22/05/2016

(date)

ACKNOWLEDGMENTS

First of all, I would like to thank the University of Tasmania and IMAS for giving me the opportunity to undertake my PhD in Australia. All the members of my supervisory team are thanked for being simply the best people you can work with. Everyone has heavily contributed to this research. Dr Jennifer Cobcroft is thanked in particular for believing in me and deciding to take me as PhD student under her supervision. Thanks so much again Jenny, you are a lovely person and you have been a fantastic and supportive mentor and I have learned so much from you. Dr Tomer Ventura has been throughout my PhD not only a superb supervisor but also a great friend. Thanks again Tomer for your friendship, valuable support and for teaching me so many things that made possible the production of this thesis. Dr Mark Adams has had a fundamental supervisory role during my PhD as he taught me all the practical and theoretical aspects of aquaculture and assisted during the experiments with his precious knowledge. Thanks Mark for your patience and for being always so nice and helpful. Last but not least, Prof Chris Carter is thanked for the incredible support provided in every possible aspect (scientific, technical and human) during this experience although being very busy for obvious reasons. Thanks Chris for being such present supervisor, you are one of the politest and nicest people I have ever met and under your guidance I have definitely become a better scholar. Special thanks also to Prof Abigail Elizur which has supervised me during the first months of my PhD and made possible my collaboration with the University of the Sunshine Coast allowing me to visit and work several times during my PhD in such fantastic university and amazing location. Thanks a lot Abigail, you have done so much for me and you are simply lovely. Thanks also to Prof Stephen Battaglene for his initial contribution during my PhD and for being always very kind with me. All technical staff at IMAS aquaculture facilities in Launceston and Taroona and at the Genecology Research Centre at USC as well as people from the Tasmanian Atlantic salmon industry are thanked for the support. Thanks to all the beautiful friends I have met here in Tasmania for being part of this long adventure. Thanks to my family for being present, supportive and for being happy for me to do this experience although very far from home.

The biggest thanks, from the bottom of my heart, to my amazing partner Ylenia. She is the one that convinced me to undertake this journey, supported me the most and shared with me the good and bad times. Thanks for loving me so much and for being the best partner I could wish for. Ti Amo!

TABLE OF CONTENTS

DECLARATION OF ORIGINALITY	III
AUTHORITY OF ACCESS	IV
STATEMENT REGARDING PUBLISHED WORK	V
CO-AUTHORSHIP	VI
STATEMENT OF ETHICAL CONDUCT	VII
ACKNOWLEDGMENTS	VIII
LIST OF FIGURES	XIII
LIST OF TABLES	XVI
ABSTRACT.....	XVII
CHAPTER 1: GENERAL INTRODUCTION	1
1.1 Atlantic salmon life history.....	2
1.2 Atlantic salmon aquaculture	3
1.2.1 Atlantic salmon aquaculture in Tasmania	4
1.3 Triploid Atlantic salmon.....	6
1.3.1 Triploid Atlantic salmon in Tasmania.....	8
1.3.2 Diploids vs Triploids: differences and similarities	9
1.4 Skeletal anomalies in Atlantic salmon.....	12
1.4.1 Triploidy and skeletal anomalies.....	15
1.4.2 Lower jaw deformity in Atlantic salmon	16
1.5 Teleosts skeletal system.....	19
1.5.1 Skeletal tissues: structure and main types	19
1.5.2 Cellular and molecular mechanisms underlying bone and cartilage formation and resorption.....	22
1.6 Aims of the research	26
CHAPTER 2: Skeletal anomaly assessment in diploid and triploid juvenile Atlantic salmon (Salmo salar L.) and the effect of temperature in freshwater	28
2.1 Abstract.....	29
2.2 Introduction.....	29
2.3 Materials and methods	31
2.3.1 Fish stock.....	31
2.3.2 Embryo incubation	32
2.3.3 Viability rate assessment.....	33
2.3.4 Experiment 1: early rearing (alevin – fry).....	33

2.3.5 Experiment 1: sampling	34
2.3.6 Experiment 2: juvenile rearing (parr/pre-smolt) at two temperatures	35
2.3.7 Ploidy determination	36
2.3.8 Experiment 2: sampling	37
2.3.9 Identification of skeletal anomalies.....	38
2.3.10 Radiological assessment.....	38
2.3.11 Statistical analysis	39
2.4 Results.....	40
2.4.1 Mortality – incubation.....	40
2.4.2 Mortality and growth performance – Experiment 1	40
2.4.3 Mortality and growth performance – Experiment 2.....	40
2.4.4 Prevalence of skeletal anomalies – Experiment 1	42
2.4.5 Prevalence of skeletal anomalies – Experiment 2	42
2.4.6 Radiological assessment – Experiment 2	45
2.5 Discussion.....	47
2.5.1 Mortality.....	47
2.5.2 Growth.....	47
2.5.3 Prevalence of skeletal anomalies.....	48
2.6 Conclusions.....	56
2.7 Acknowledgments	57
CHAPTER 3: Concurrence of lower jaw skeletal anomalies in triploid Atlantic salmon (<i>Salmo salar</i> L.) and the effect on growth in freshwater	58
3.1 Abstract.....	59
3.2 Introduction.....	59
3.3 Materials and methods	62
3.3.1 Fish source.....	62
3.3.2 Experiment design and conditions	63
3.3.3 Visual assessment of lower jaw skeletal anomalies	64
3.3.4 Growth and lower jaw index (LJI)	66
3.3.5 Statistical analysis	66
3.4 Results.....	66
3.4.1 Fish performance.....	66
3.4.2 Prevalence of lower jaw skeletal anomalies.....	67
3.4.3 Concurrence of lower jaw skeletal anomalies and types.....	68

3.4.4 Lower jaw index (LJI).....	70
3.4.5 Opercular shortening	70
3.5 Discussion.....	72
3.6 Conclusions.....	78
3.7 Acknowledgements.....	79
CHAPTER 4: Multigenic delineation of lower jaw deformity in triploid Atlantic salmon (<i>Salmo salar</i> L.).....	80
4.1 Abstract.....	81
4.2 Introduction.....	81
4.3 Materials and methods	83
4.3.1 Sample background, selection and tissue source	83
4.3.2 RNA extraction and preparation for next generation sequencing	85
4.3.3 Next generation sequencing and data handling.....	85
4.3.4 Bioinformatics and statistics	86
4.3.5 cDNA synthesis, probe and primer design for qPCR.....	87
4.3.6 Real-time qPCR assays	88
4.3.7 General Histology	90
4.3.8 H&E staining.....	90
4.3.9 In-situ Hybridization	90
4.4 Results.....	92
4.4.1 Transcriptome analysis of the experimental sample set.....	92
4.4.2 Down-regulated transcripts	93
4.4.3 Up-regulated transcripts	94
4.4.4 Real-time qPCR validation	96
4.4.5 Real-time qPCR of different developmental stages	97
4.4.6 Real-time qPCR for fish reared at different temperatures (14 vs 18 °C)	98
4.4.7 GPHB5 In-Situ Hybridization.....	98
4.5 Discussion.....	99
4.6 Conclusions.....	106
4.7 Acknowledgements.....	107
CHAPTER 5: GENERAL DISCUSSION.....	108
5.1 General discussion	109
5.1.1 LJD development clues, the effect of ploidy and temperature on skeletal anomaly occurrence and considerations on opercular shortening	110

5.1.2 Concurrence and worsening of lower jaw anomalies, the effect of growth rate, opercular shortening recovery and deleterious effect of lower jaw anomalies	114
5.1.3 Considerations on lower jaw plasticity	117
5.1.4 Triploids and dietary requirements	117
5.1.5 Molecular characterisation of LJD and detection of possible markers	118
REFERENCES	121

LIST OF FIGURES

- Fig. 1.1. Atlantic salmon (*Salmo salar*) lifecycle. **Page 3**
- Fig. 1.2. Production cycle of Atlantic salmon (*Salmo salar*). **Page 5**
- Fig. 1.3. Osteoblastogenesis. **Page 25**
- Fig. 1.4. Osteoclastogenesis. **Page 25**
- Fig. 2.1. Wet weight during first experiment of triploid and diploid Atlantic salmon *Salmo salar* fry/parr. **Page 41**
- Fig. 2.2. Wet weight during the second experiment of triploid and diploid Atlantic salmon *Salmo salar* parr/pre-smolt for standard (14 °C) elevated (18 °C) treatments. **Page 41**
- Fig. 2.3. Prevalence of skeletal anomalies in diploid and triploid Atlantic salmon *Salmo salar* fry/parr observed during last two samplings of first experiment. **Page 42**
- Fig. 2.4. Prevalence of skeletal anomalies in diploid and triploid Atlantic salmon *Salmo salar* parr/pre-smolt observed throughout the second experiment for standard (14 °C) and elevated (18 °C) treatments. **Page 44**
- Fig. 2.5. Prevalence of skeletal anomalies in Atlantic salmon *Salmo salar* parr/pre-smolt observed during the last sampling at the same age (~3400° days) in both standard (14 °C) and elevated (18 °C) treatments. **Page 45**
- Fig. 2.6. Regions of the vertebral column with deformed vertebrae in diploid and triploid Atlantic salmon *Salmo salar* pre-smolt observed at the end of the second experiment in standard treatment (14 °C) and elevated treatment (18 °C). **Page 46**
- Fig. 3.1. Atlantic salmon (*Salmo salar* L.) parr (approximately 12 g) displaying shortened lower jaw (SJ) and a concurrence of shortened lower jaw (SJ) and lower jaw deformity (LJD). **Page 63**
- Fig. 3.2. Classification and severity of lower jaw skeletal anomalies affecting Atlantic salmon (*Salmo salar* L.) individuals. **Page 65**
- Fig. 3.3. Weight and fork length of Atlantic salmon (*Salmo salar* L.) individuals in the two groups NOR and SJ (established at the beginning of the experiment according to the jaw trait initially displayed - normal or shortened lower jaw) during the four visual assessments performed. **Page 67**
- Fig. 3.4. Mean prevalence of lower jaw anomaly categories (considered individually) observed in Atlantic salmon (*Salmo salar* L.) individuals within the two groups NOR and SJ during the four visual assessments performed. **Page 68**

Fig. 3.5. Mean prevalence of Atlantic salmon (*Salmo salar* L.) individuals displaying no anomalies (or normal) and one or more lower jaw skeletal anomalies concurrently within the two groups NOR and SJ during the four visual assessments performed.

Page 69

Fig. 3.6. Mean prevalence of lower jaw skeletal anomaly categories displayed concurrently in Atlantic salmon (*Salmo salar* L.) individuals, within the fish that had two or more concurrent anomalies, in the two groups NOR and SJ during the four visual assessments performed. **Page 70**

Fig. 3.7. Atlantic salmon (*Salmo salar* L.) (approximately 60 g) displaying a concurrence of severe shortened lower jaw (SJ) and mild lower jaw deformity (LJD) at the end of the experiment. **Page 70**

Fig. 3.8. Prevalence of opercular shortening in Atlantic salmon (*Salmo salar* L.) individuals in the two groups NOR and SJ during the experiment. **Page 71**

Fig. 3.9. The operculum of Atlantic salmon (*Salmo salar* L.) individuals at same stage of development showing different degrees of recovery from opercular shortening. **Page 71**

Fig. 4.1. a) Atlantic salmon individuals displaying a normal jaw and a lower jaw deformity (LJD). b) Hierarchical clustering showing differentially expressed transcripts between Normal vs. LJD grouped samples retrieved after a pairwise comparison.

Page 93

Fig. 4.2. Relative expression of selected transcripts found to be differentially expressed in two different categories (Normal and affected by lower jaw deformity, LJD) after transcriptome analysis of triploid Atlantic salmon *Salmo salar* pre-smolts from the experimental sample set. **Page 96**

Fig. 4.3. a) Relative expression of the eleven transcripts tested in triploid Atlantic salmon *Salmo salar* individuals belonging to two independent sample sets, ‘experimental’ (60 g) and ‘industrial’ (100 g) and displaying a normal lower jaw (Normal) or a lower jaw deformity (LJD). b) A graphic summary of the previous graphs to show significantly different regulation and corresponding approximate fold change in LJD individuals only relative to Normal from the two independent sample sets. **Page 97**

Fig. 4.4. Relative expression of *col2a1* in Normal and lower jaw deformity (LJD) affected triploid Atlantic salmon *Salmo salar* from the experimental sample set at standard (14 °C) and elevated (18 °C) temperature. **Page 98**

Fig. 4.5. A transverse H&E stained section of the lower jaw of a triploid Atlantic salmon (~60 g) affected by lower jaw deformity (LJD) and in-situ hybridization stained sections of a LJD affected jaw for *gphb5*. **Page 99**

LIST OF TABLES

Table 2.1. Sampling events and the equivalent age in °C days (cumulative average daily temperature) of diploid and triploid Atlantic salmon parr/pre-smolts kept in different temperature treatments (Standard 14 °C and Elevated 18 °C) sampled throughout the experiment. *Page 37*

Table 4.1. Primers and probes used for real-time qPCR designed from previously known transcripts whose function was annotated to be related to bone and cartilage physiology. *Page 89*

Table 4.2. Primers and probes used for real-time qPCR from selected transcripts found differentially expressed after transcriptome analysis in the experimental sample set. *Page 89*

Table 4.3. The six transcripts found down-regulated in LJD selected according to Sum of RPKM ≥ 5 across all 4 pooled samples ordered by E-value of the best hit after transcriptome analysis. *Page 94*

Table 4.4. The 27 transcripts found up-regulated in LJD selected according to Sum of RPKM ≥ 5 across all 4 pooled samples ordered by E-value of the best hit after transcriptome analysis. *Page 95-96*

ABSTRACT

Triploid Atlantic salmon are a valuable part of production in Tasmania. In fact, not undergoing sexual maturation, triploids are used to ensure appropriately sized harvest fish all year round. Nevertheless, triploids tend to develop a higher prevalence of skeletal anomalies than diploids affecting mainly the lower jaw and the vertebral column and impacting fish performance, welfare and value deleteriously. The general intent of this PhD research project was to make a useful contribution to Atlantic salmon industry in order to understand and find a possible solution or mitigate the problem of skeletal anomalies of obvious relevance to economic efficiency as well as animal welfare in Tasmania and worldwide. This research explored the occurrence of several skeletal anomalies in farmed diploid and triploid Atlantic salmon in freshwater that represent a critical phase in development. The aims were to improve our knowledge on occurrence and causative factors of skeletal anomalies in triploids (and diploids) and in particular of lower jaw deformity (LJD), a skeletal anomaly mainly affecting triploid Atlantic salmon, and to understand the molecular mechanisms underlying it. Two experiments (the first containing two sub-experiments) and an extensive molecular investigation have been undertaken during this PhD research project in order to produce valuable information and fulfil the predetermined aims.

In the first experiment, diploids and triploids were maintained in controlled conditions for nine months (from incubation to 60 g) and repeatedly screened (visually and by x-ray) for the occurrence of several categories of skeletal anomalies (i.e. lower jaw anomalies, opercular shortening, spinal anomalies) and the effect of standard and elevated temperature regimes (14 and 18 °C) on prevalence of skeletal anomalies was tested in the pre-smolt stage (8-60 g). This experiment showed that LJD started to be displayed at 8-10 g and the prevalence increased only in triploids up to 11% at 60 g implying that occurrence can be at any time in development. In diploids, LJD prevalence was approaching to zero throughout the experiment. A high

prevalence of opercular shortening was observed (average prevalence in both ploidies 85.8%) and shortened lower jaw (SJ) was common in both ploidies (highest prevalence observed 11%). In triploids, the change in prevalence over time of LJD and SJ indicated a possible developmental link between the two jaw anomalies. No effect of temperature was observed on prevalence of lower jaw skeletal anomalies. A radiological assessment ($n = 240$ individuals) showed that at both temperature regimes tested triploids had a significantly ($P < 0.05$) lower number of vertebrae and higher prevalence of deformed individuals and that elevated temperature resulted in more regions of the vertebral column affected irrespective of the ploidy. Our findings confirmed that LJD occurs predominantly in triploids and at any time during freshwater phase and overall ploidy was more influential than temperature on development of skeletal anomalies.

The second experiment, developed following the results of the first experiment, focused on LJD developmental patterns in triploids and LJD concurrence with another lower jaw anomaly affecting Atlantic salmon, shortened lower jaw (SJ). Triploid individuals (~12 g) only were collected and selected at a commercial hatchery, allocated into two separate groups according to their lower jaw condition (normal -NOR- or affected by SJ -SJ-), grown out in controlled conditions and assessed (visually and by image analysis) over three months for concurrence of SJ and LJD. The main aim of the experiment was to verify concurrence of lower jaw skeletal anomalies in the two groups and understand through their developmental patterns independency or dependency of the conditions. Furthermore, effect of lower jaw anomalies on growth and recovery from opercular shortening was tested. Prevalence of jaw anomalies increased in both groups over time (NOR group 0 to 24.5%; SJ group 17 to 31%). SJ and LJD occurred both independently and concurrently whereas another lower jaw anomaly, misaligned lower jaw (MA), concurred exclusively with them. All three anomalies could be concurrent. Severity of both LJD and SJ increased in the SJ group only. Opercular shortening recovery was

observed in both groups but at a slower rate in the SJ group. The SJ group specific growth rate (SGR) was significantly ($P < 0.05$) lower than the NOR group. This study demonstrated the concurrence of SJ, LJD (and MA) in triploids and showed possible deleterious consequences deriving from the condition/s.

The last research chapter, describes the first molecular analysis ever undertaken on fish affected by LJD. Two independent sample sets of triploid Atlantic salmon, experiment-sourced (60 g) (from the first experiment) and commercially produced (100 g) displaying LJD and with a normal lower jaw were used to delineate multigenicity of the condition. The main aim was to investigate, by using transcriptome analysis, real-time qPCR and *in-situ* hybridization, molecular mechanisms underlying the condition and to detect possible candidate genes providing clues on tissue/s responsible for the trait. The use of two independent sample sets at different developmental stages helped to strengthen the outcome of the analyses and verify differential gene expression consistency. The effect of temperature on gene regulation was also tested. A total of eleven genes, some detected through transcriptome analysis (*fhn2*, *gal* and *gphb5*) and others known to be related to skeletal physiology (*alp*, *bmp4*, *colla1*, *col2a1*, *fgf23*, *igf1*, *mmp13*, *ocn*), were tested in the two independent sample sets. *Gphb5*, a recently discovered hormone, was significantly ($P < 0.05$) down-regulated in LJD affected fish in both sample sets suggesting a possible hormonal involvement. *In-situ* hybridization detected *gphb5* expression in oral epithelium, teeth and skin of the lower jaw. *Col2a1* showed the same consistent significant ($P < 0.05$) down-regulation in LJD suggesting a possible cartilaginous impairment as a distinctive feature of the condition. Significant ($P < 0.05$) differential expression of other genes found in either one or the other sample set highlighted the possible effect of stage of development or condition progression on transcription and showed that anomalous bone development, likely driven by cartilage impairment, is more evident at larger fish sizes. The present study improved our understanding of LJD suggesting that a cartilage

impairment likely underlies the condition and *col2a1* may be a marker. In addition, the involvement of *gphb5* urges further investigation of a hormonal role in LJD and skeletal physiology in general.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Atlantic salmon life history

Atlantic salmon (*Salmo salar* L. 1758) belongs to the Salmonidae family and is one of the most known and studied species of fish. The species is found in the North Atlantic Ocean on the coasts of both Europe and North America between 40° and 70°N (Gross, 1998; Jobling *et al.*, 2010). An essential feature of the species is the anadromous life cycle: it spends a part of the life cycle in freshwater (spawning, initial growth and feeding) and a part in seawater (further feeding and growth until sexual maturity) before returning to freshwater to spawn (Fig. 1.1) (Gross, 1998; Marschall *et al.*, 1998; Jobling *et al.*, 2010). Atlantic salmon, like other salmonids, displays homing behaviour, which means that individuals return to spawn in the rivers where they were born or previously spawned (Gross, 1998; Marschall *et al.*, 1998; Jobling *et al.*, 2010). In addition, Atlantic salmon is iteroparous meaning that after the first spawning individuals do not die and are able to spawn again the following year (Klemetsen *et al.*, 2003). Sexual maturity is reached at 3-6 kg and 1-3 years spent in the seawater (Jobling *et al.*, 2010). A female salmon generally produces about 1500 eggs kg⁻¹ body weight although the number is variable (Jobling *et al.*, 2010). Immediately after fertilisation, the females bury the eggs in gravel for incubation into a nest called 'redd' (Jobling *et al.*, 2010). Eggs measure 5-6 mm in diameter and hatch after several months depending on water temperature, usually two months post-fertilisation (Jobling *et al.*, 2010). Hatched individuals are called alevins (15-25 mm total length) and have a large yolk-sac that provides endogenous energy requirements. Once the yolk-sac has been resorbed alevins are ready to feed and leave the redd. This stage is called the 'swim-up' phase and the individuals are identified now as fry. From the fry stage, individuals develop into parr. Parr stage can last for several years until fish are ready to become smolts (smoltification process), acquiring the adequate physiological and morphological characteristics (e.g. changes in osmoregulation and body colour) to live in the saltwater (Gross, 1998; Jobling *et al.*, 2010).

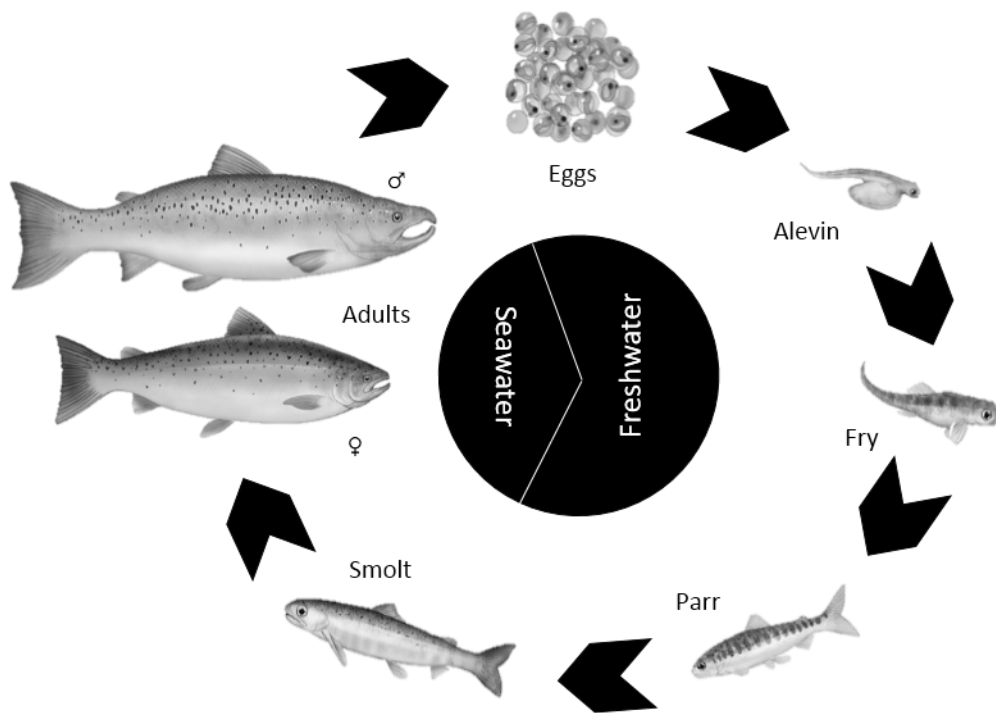


Fig. 1.1. Atlantic salmon (*Salmo salar*) lifecycle
(modified from <http://www.miramichisalmon.ca/education/atlantic-salmon/> and Jobling *et al.*, 2010)

1.2 Atlantic salmon aquaculture

During the last part of the 20th century Atlantic salmon aquaculture thrived and is currently spread in numerous parts of the world such as northern Europe, North and South America and Australasia (Gross, 1998; Jobling *et al.*, 2010; FAO, 2016a). The cultivation techniques are similar in all the countries where the industry is established (Gross, 1998; Jobling *et al.*, 2010). Briefly, the general production cycle includes the following phases: incubation of eggs, early rearing and smolt production in both freshwater hatcheries and smolt production units, transfer to seawater and on-growing to market size (3-7 kg), a phase that is dominated by sea-cage culture (Gross, 1998; Jobling *et al.*, 2010). For a more comprehensive description of the production cycle refer to Fig. 1.2. The production of farmed Atlantic salmon in 2013 was 2,087,111 tonnes and valued at around USD 13 billion (FAO, 2016b). Norway is the most

important producer with 1,168,324 tonnes (~60% of total production) followed by Chile 492,329 tonnes (~24%), United Kingdom 154,200 tonnes (~7%), Canada 100,027 tonnes (~5%), Australia 42,776 tonnes (~2%) and United States of America 18,685 tonnes (~1%) (FAO, 2016b).

1.2.1 Atlantic salmon aquaculture in Tasmania

Atlantic salmon farming started in Tasmania at the end of 1984 (Jungalwalla, 1991; Ovenden *et al.*, 1993). The first fertilised eggs shipped to Tasmania came from a landlocked broodstock kept in Gaden, New South Wales, where between 1963 and 1965 Atlantic salmon were imported into Australia from Nova Scotia, Canada for the first time (Jungalwalla, 1991). Soon after the first attempts at farming, Tasmania was identified as a favourable place for Atlantic salmon due to the relatively high seawater temperatures that enhanced growth (Jungalwalla, 1991). An additional advantage for farming Atlantic salmon in Tasmania was that the species, not being farmed in its natural range, would not have any contact with wild strains, avoiding infectious disease problems and possible ecological and negative biological interactions (Benfey, 2015b; Benfey, 2015a; Glover *et al.*, 2016).

Currently, the Tasmanian salmon industry is Australia's largest and most valuable aquaculture industry. According to the latest data available, it produced ~40,000 tonnes in 2013-14 (AUD 531.3 million, including exports AUD ~14 million) (Stephan and Hobsbawn, 2015). Salmonids aquaculture, predominantly Atlantic salmon from Tasmania, represents ~55% of the total value of Australian aquaculture production and ~22% of the total value of fisheries and aquaculture production (Stephan and Hobsbawn, 2015), with plans to expand further in the future (TSGA - Tasmanian Salmonid Growers Association, unpublished data). Nevertheless, numerous challenges confront the industry including control and management of disease (including skeletal anomalies), suitable locations to farm, competition with foreign producers

and climate change that may limit the growth of the Atlantic salmon industry (Battaglene *et al.*, 2008; Hobday *et al.*, 2008; Carter *et al.*, 2010).

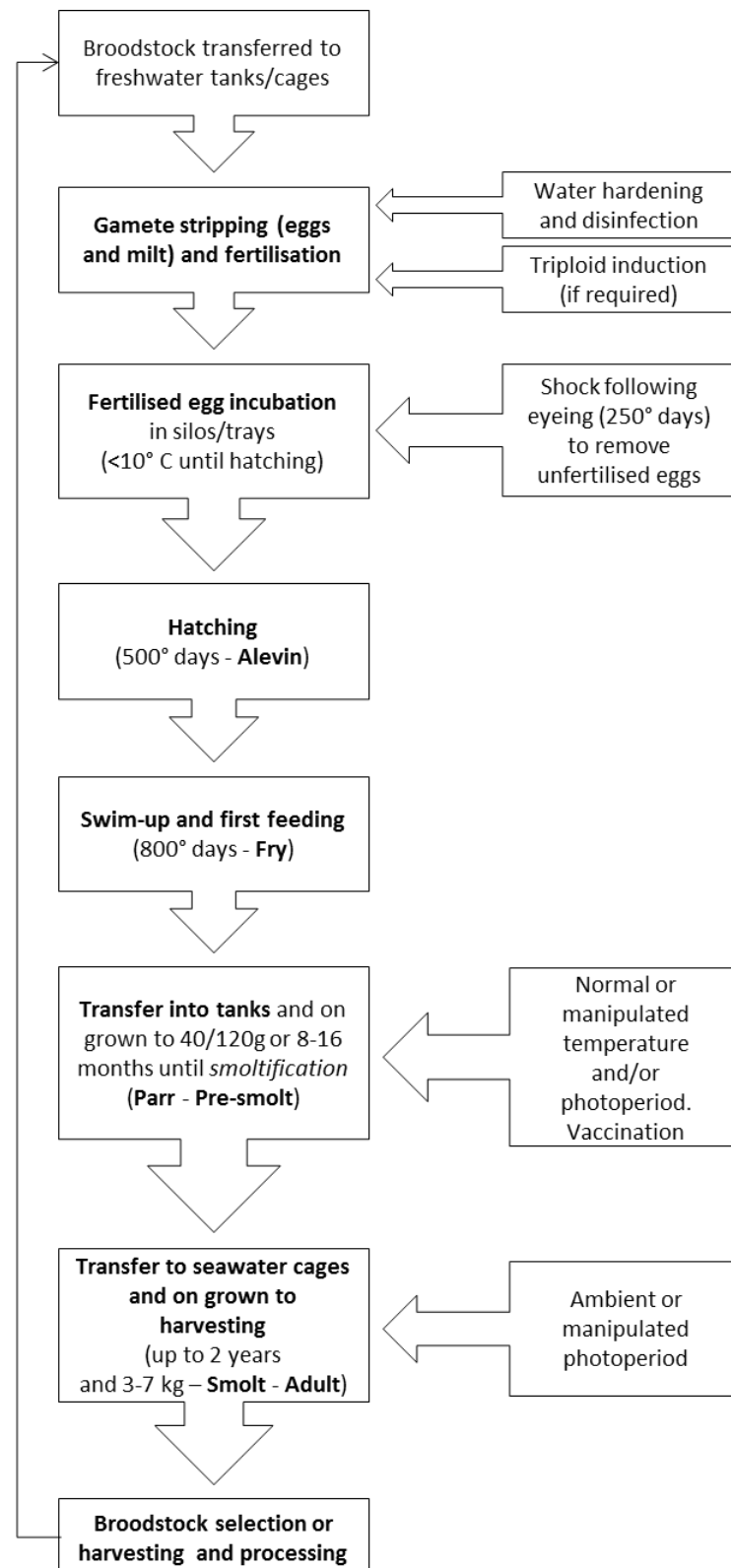


Fig. 1.2. Production cycle of Atlantic salmon (*Salmo salar*)

1.3 Triploid Atlantic salmon

A crucial obstacle for the continuity of the Atlantic salmon production cycle is presented by early sexual maturation (Sadler, 2000; Fraser *et al.*, 2012a; Benfey, 2015b). In fish development, maturation represents a critical phase having several deleterious effects relevant to aquaculture: fish usually display slower growth because energy is depleted due to the high metabolic costs of gametogenesis, they are more susceptible to diseases and flesh quality is reduced (Benfey, 1999; Sadler *et al.*, 2000b; Oppedal *et al.*, 2003; Piferrer *et al.*, 2009; Taranger *et al.*, 2010; Benfey, 2015b).

In order to avoid sexual maturation, the practice of triploidy induction was chosen in fish farming, including salmonid production, as a method to sterilise a large number of fish without the use of chemicals, and subsequently to obtain a product more acceptable by the market (Benfey, 2001; 2015b). In regions where there are wild populations the use of sterile Atlantic salmon also has the advantage of preventing possible interbreeding between wild and farmed populations, and associated effects on the ecosystem deriving from accidental escapes (Sadler *et al.*, 2000b; Benfey, 2001; Oppedal *et al.*, 2003; Taylor *et al.*, 2011; Benfey, 2015b). Nevertheless, triploid Atlantic salmon are currently, as they have for many years, used for commercial production only in Australia (Tasmania) (Benfey, 2015b) although only recently (May 2016) the production of the first triploid populations was approved in Norway (<http://www.reuters.com/article/idUSO9N17G00Y>). However, in both Scotland and Norway, triploids are still under investigation for utilisation in the production cycle due to the reasons described in the following paragraphs (Leclercq *et al.*, 2011; Fraser *et al.*, 2013; Taylor *et al.*, 2013; Taylor *et al.*, 2014; Benfey, 2015b; Fjellidal *et al.*, 2016). It should be noted that in this thesis Atlantic salmon will be referred to as diploid (as the normal genome situation) to improve the readability although it has been recently shown that the species is actually pseudotetraploid

due to a genome duplication event occurring ~ 80 million years ago in the salmonid lineage (Lien *et al.*, 2016).

Triploidy is artificially achieved by applying a shock (chemical, thermal or pressure) to eggs during meiosis II that suppresses cell division and prevents the extrusion of a polar body interfering with the regular movements of the chromosomes (Benfey, 2001; Piferrer *et al.*, 2009; Fraser *et al.*, 2012a; Benfey, 2015b). In particular for Atlantic salmon, pressure shock has been preferred to thermal shock to induce triploidy due to insufficient results with the latter method (i.e. variable results of triploidy effectiveness and higher mortality rates) (Sutterlin *et al.*, 1987; Jungalwalla, 1991) relative to better results obtained using pressure shock (Benfey and Sutterlin, 1984b; Johnstone *et al.*, 1991; Jungalwalla, 1991; Benfey, 2015b). Therefore, to produce triploid Atlantic salmon, the pressure shock is usually delivered at 30 minutes post fertilisation, when eggs are kept at 10 °C, at 9500 psi (655 Bar) and lasts 5 minutes (Johnstone *et al.*, 1991; Jungalwalla, 1991; Johnstone and Stet, 1995; Benfey, 2015b). Nevertheless, induction time and length may vary depending on water temperature and consequent embryonal stage of development (Benfey, 2015b). Chemical triploidy induction has not been considered for Atlantic salmon because of the method's unreliability and, as said above, the possible negative consequences related to market acceptance (Jungalwalla, 1991; Benfey, 2015b). Interestingly, in Norway it has been recently shown that triploidy can occur spontaneously in diploid populations at an average rate of ~2% as a consequence of husbandry practices (Glover *et al.*, 2015).

Triploid organisms have, in each somatic cell of the body, three sets of chromosomes instead of two (Benfey, 2001). In Atlantic salmon, there are several biological effects that originate from artificial triploidy (Allendorf and Leary, 1984; Benfey, 2001), in light of the above, first and most important is that the fish are sterile due to gametogenic impairment and its effects on gonadal development. Second, the individuals display larger cells (increased

cellular volume to contain extra genetic material) and a smaller number of cells (and consequently, not a larger body size). Third, triploids have increased heterozygosity compared to diploids.

There are several possible biological consequences of triploidy induction. Among them, the increased cellular volume could have effects on some fundamental physiological processes, such as nutrients and metabolites exchange, passive and active ion exchange, and membrane binding of hormones and other messengers due to a decrease in the ratio of surface area to volume (as reviewed by Benfey, 1999; 2015b). In addition, the lower cell numbers combined with a decrease in ratio of surface area to volume may represent a problem not only for the functions of the single cell but also for tissues and organs. Another relevant effect, caused by an increase in size of the cells and the nuclei, could be on the transport and diffusion rates across membranes and in particular on signal transduction (e.g. involving RNA and protein production and movement) (Benfey, 1999; 2015b). Nevertheless, according to Maxime (2008) there is still no certainty whether an increased cellular volume and a decrease in ratio of surface area to volume may effectively lead to the physiological dysfunctions mentioned above.

1.3.1 Triploid Atlantic salmon in Tasmania

In Tasmania, the Atlantic salmon industry has applied sterilisation to inhibit earlier sexual maturation, caused by higher temperatures compared to the northern hemisphere, leading to a reduction in duration of the harvest period (Sadler, 2000; Benfey, 2001; Benfey, 2015b). The total amount of triploid fish in Tasmanian production is estimated to be less than 10% according to Sadler (2000), but recently it has increased to 20% with the possibility of increasing further due to industry expansion in Macquarie Harbour on the west coast of Tasmania, where all-female triploids are the primary stock type (unpublished data).

Three different population types of Atlantic salmon are produced in Tasmania: normal or mixed sex diploids, all-female diploids and all-female triploids (Sadler *et al.*, 2000b). In

addition, photoperiod-manipulated fish are also produced (Sadler, 2000). All-female populations are produced using milt from masculinised females, functional males with the XX-genotype (also known as neomales or sex-reversed females), for fertilisation in order to obtain female zygotes (Benfey, 2015b). The sex reversal process is achieved by dietary addition of 17 α -methyltestosterone during the fry/parr stage and these hormone-fed fish are never destined for harvest and human consumption, only as the source of gametes (Jungalwalla, 1991; Benfey, 2001; Benfey, 2015b). The industry uses all-female populations in order to avoid early maturation and secondary sexual characteristics of salmon males (compared to females) that are not suitable for the market due to aesthetic reasons and substantially reduced flesh qualities (Sadler *et al.*, 2000b; Benfey, 2015b). In fact, the male counterparts, also when sterile, undergo the normal process of sexual maturation (Benfey, 2001; Benfey, 2015b). As mentioned above, these different populations are used to extend the period of harvest or fill the gaps in between (Sadler, 2000). The role of all-female triploid populations of Atlantic salmon is crucial for production cycle continuity in Tasmania providing a year-round supply of fresh product (Jungalwalla, 1991; Sadler, 2000).

1.3.2 Diploids vs Triploids: differences and similarities

According to Benfey (2001), triploid fish should be considered a ‘different species’ from the diploid counterparts due to differences in performance under farming conditions. Comprehensive reviews have been published on the differences and similarities in physiology and culture requirements between diploids and triploids (Benfey, 1999; Maxime, 2008; Piferrer *et al.*, 2009; Fraser *et al.*, 2012a; Benfey, 2015b). With regards to triploid Atlantic salmon, an extensive literature has accumulated over the last 25 years investigating several aspects concerning triploid use in the production cycle.

Survival rate of triploid Atlantic salmon has been shown to differ between countries, years, populations, stage of development (freshwater or seawater) and if reared communally

with diploids or in isolation. For instance, diploids and triploids showed no difference in survival rate when reared separately (Carter *et al.*, 1994; Oppedal *et al.*, 2003; Fjelldal and Hansen, 2010; Leclercq *et al.*, 2011; Taylor *et al.*, 2011; Taylor *et al.*, 2012; Taylor *et al.*, 2013; Taylor *et al.*, 2014) or in cohabitation (Carter *et al.*, 1994; McGeachy *et al.*, 1996; O'Flynn *et al.*, 1997; Taylor *et al.*, 2014), while in other cases triploids showed either reduced (Cotter *et al.*, 2002) or increased (Fraser *et al.*, 2015) survival rates compared to diploids when reared separately. Nevertheless, generally triploids showed reduced survival rates relative to diploids when reared together likely due to differences in behaviour and feed utilisation (Quillet and Gaignon, 1990; Galbreath *et al.*, 1994; Galbreath and Thorgaard, 1995; McCarthy *et al.*, 1996; O'Flynn *et al.*, 1997; Fjelldal and Hansen, 2010).

Similar variable results have been reported with regards to growth. Several studies found that triploids grew similarly (Benfey and Sutterlin, 1984a; Quillet and Gaignon, 1990; Jungalwalla, 1991; Carter *et al.*, 1994; Galbreath *et al.*, 1994; McGeachy *et al.*, 1995; Cotter *et al.*, 2002) or faster (O'Flynn *et al.*, 1997; Oppedal *et al.*, 2003; Leclercq *et al.*, 2011; Fraser *et al.*, 2013; Taylor *et al.*, 2013) compared to diploids being reared separately in freshwater. Similar growth rates among ploidies have also been observed by several authors in seawater (Jungalwalla, 1991; Galbreath and Thorgaard, 1995; O'Flynn *et al.*, 1997; Cotter *et al.*, 2002; Leclercq *et al.*, 2011; Sacobie *et al.*, 2012; Taylor *et al.*, 2013). Only recently triploids have been shown to have reduced growth in seawater at harvest compared to diploids (Taylor *et al.*, 2014; Fraser *et al.*, 2015) but the opposite occurred when triploids were fed a diet with higher content of dietary proteins and phosphorous (Smedley *et al.*, 2016).

Other similarities between the two ploidies have also been found regarding performance under stressful conditions (i.e. fish subjected to confinement both in freshwater and seawater) in particular in the haematological response (e.g. same levels of plasma glucose, lactate and cortisol), in oxygen carrying capacity (Sadler *et al.*, 2000b; Sadler *et al.*, 2000a) and in the

smolting rate under normal conditions (Quillet and Gaignon, 1990; Cotter *et al.*, 2002; Taylor *et al.*, 2014).

Differences between ploidies have been found in the smolting rate only in studies with “out-of-season” smolts (produced by photoperiod manipulation) in which triploid smolts completed smoltification four weeks earlier than diploids reared separately (Leclercq *et al.*, 2011; Taylor *et al.*, 2012). Furthermore, triploids perform inadequately compared to diploids when reared in suboptimal conditions. In particular Quillet and Gaignon (1990) and Johnstone *et al.* (1991) found that triploid salmon subjected to low level of dissolved oxygen performed inadequately compared to diploids, and Galbreath and Thorgaard (1995) found a lower condition factor and growth rate in triploids compared to diploids farmed in seawater net-pen. Cotterell and Wardle (2004) found differences between ploidies in the anaerobic capacity, lower in triploids, but not in the aerobic capacity during forced swimming. On the other hand, triploid Atlantic salmon displayed a better recovery than diploids considering the oxygen consumption after exercise (Lijalad and Powell, 2009). In addition, significant differences were found in metabolic rates: triploids showed higher rates than diploids at lower temperatures (12 °C) and the opposite occurred at higher temperatures (18 °C) (Atkins and Benfey, 2008). In support of that, Hansen *et al.* (2015) reported poorer performance of triploids relative to diploids exposed to high temperature (19 °C) for an extended period and subjected to moderate hypoxia.

Recently, other critical differences between ploidies have been discovered. Fraser *et al.* (2012b), analysing the size of different parts of the brain in triploids and diploids, found a smaller olfactory bulb and a larger cerebellum and telencephalon in triploids relative to diploids implying behavioural differences between ploidies. Taylor *et al.* (2015) showed that triploids have higher requirements of histidine that plays a fundamental role in preventing ocular cataracts. Finally, Fjelldal *et al.* (2016) and Smedley *et al.* (2016) have shown that triploids

have different requirements of dietary phosphorus (P) relative to diploids, during both freshwater and seawater phase, impacting both growth (lower in triploids than diploids) and occurrence of skeletal anomalies (higher in triploids than diploids). These results urge industry and feed producers to take into consideration these factors in order to avoid repercussions on triploids welfare and more specifically skeletal development in the case of P requirements.

Finally, one of the most critical differences between ploidies, that is currently limiting the use of triploid fish in other countries, is the higher incidence and prevalence of skeletal anomalies in triploids compared to diploids (Benfey, 2001; Maxime, 2008; Piferrer *et al.*, 2009; Fraser *et al.*, 2012a; Fraser *et al.*, 2013; Taylor *et al.*, 2013; Benfey, 2015b).

1.4 Skeletal anomalies in Atlantic salmon

During the last twenty years, due to the high growth in production volume and geographical expansion of Atlantic salmon aquaculture, and the concurrent increase in demand, the principal aims of industry have been to improve the rearing conditions in conjunction with the reduction of costs and production time (Kristensen *et al.*, 2012; Ytteborg *et al.*, 2012). In all countries in which aquaculture is practised, skeletal anomalies (also referred to as deformities, abnormalities or malformations) represent one of the main issues affecting the production of high quality juveniles and leading to significant economic losses (Fjelldal *et al.*, 2012c; Boglione *et al.*, 2013a; Cobcroft and Battaglione, 2013). The development of new strategies to optimize rearing conditions (in particular temperature/light regimes and diets) is necessary in order to obtain, in the case of Atlantic salmon, smolts of high quality which grow rapidly, avoiding the economic loss deriving from the culling of deformed fish, and enhancing animal welfare (Kristensen *et al.*, 2012; Ytteborg *et al.*, 2012; Boglione *et al.*, 2013a; Cobcroft and Battaglione, 2013).

The importance of Atlantic salmon to aquaculture has led to the publication of an extensive amount of research regarding skeletal anomalies aiming to understand the causative

factors and the underlying mechanisms. Several factors have been shown to have an effect on normal skeletal development and growth, and these factors, that are routinely manipulated during the production of farmed fish, represent a significant risk to fish of developing anomalies (Fjelldal *et al.*, 2012c; Boglione *et al.*, 2013b). As reviewed by Fjelldal *et al.* (2012c), the two most important factors indicated as causative in Atlantic salmon are photoperiod manipulation (usually 24-h of light for most of the freshwater phase) and the use of high temperature to accelerate the growth. Photoperiod has a fundamental effect on physiological processes in Atlantic salmon such as smoltification, growth and sexual maturation and according to Fjelldal *et al.* (2012b) photoperiod influences vitamin D system functionality, which plays a crucial role in phosphorus absorption (Lock *et al.*, 2010), and osteoclast (bone resorbing cells) activity. The use of high temperature regimes can induce impairments in gene transcription of osteoblasts (bone forming cells) and chondrocytes (cartilage forming cells) and affect structure and composition of skeletal tissues (Ytteborg *et al.*, 2010b). Furthermore, deficiency of fundamental nutrients, especially phosphorus in specific stages, has been identified as another major causative factor (Fjelldal *et al.*, 2012c; 2016). Phosphorus, together with calcium, is the main component of bone in vertebrates and fish can assimilate it mostly through the diet. As reviewed by Lall and Lewis-McCrea (2007) osteoclasts activity may be related to phosphorus homeostasis and inadequate levels (above or below the standard requirements) of phosphorus in the body can lead to an imbalance in calcium to phosphorus ratio and consequent disorders in skeletal development. In light of the above, interactions between all these causative factors and likely other factors involved in Atlantic salmon aquaculture (e.g. genetic), enhance the probability to generate imbalances in growth and skeletal development/mineralisation time leading to the occurrence of anomalies.

The most common skeletal anomalies in farmed Atlantic salmon reported in the literature are spinal anomalies (column or vertebral bodies) (Gjerde *et al.*, 2005; Witten *et al.*,

2005; Witten *et al.*, 2006; Fjelldal *et al.*, 2007b; Fjelldal *et al.*, 2012c). As reviewed by Gjerde *et al.* (2005) vertebral anomalies (generally referred to as vertebral deformities) were reported as a problem for the first time by McKay and Gjerde (1986) and considered the cause of considerable economic losses due to the high prevalence (Asgard *et al.*, 1996; Vagsholm and Djupvik, 1998). In farmed Atlantic salmon several types of vertebral anomalies occur at all life stages and the most frequent are fusions or compressions of the vertebral bodies (Witten *et al.*, 2005; Witten *et al.*, 2006; Fjelldal *et al.*, 2007b; Witten *et al.*, 2009; Fjelldal *et al.*, 2012c). Many factors have been suggested to cause vertebral anomalies, including inflammatory processes (Kvellestad *et al.*, 2000; Gil-Martens, 2010), poor mineralisation of vertebral bodies (Fjelldal *et al.*, 2007a; Fjelldal *et al.*, 2009), malfunction of osteoblasts due to mechanical overload (Kvellestad *et al.*, 2000; Witten *et al.*, 2005; Witten *et al.*, 2006), increase in tartrate-resistant acid phosphatase (TRAP) enzyme activity in vertebral bone (Fjelldal *et al.*, 2012b), high incubation temperatures (Takle *et al.*, 2005; Wargelius *et al.*, 2005; Ytteborg *et al.*, 2010c; Fraser *et al.*, 2015), deficiency of vitamin C (ascorbic acid) (Waagbø, 2010; Moren *et al.*, 2011), low dietary phosphorus (Baeverfjord *et al.*, 1998; Fjelldal *et al.*, 2011), fast smolt production with continuous light and elevated temperature (Fjelldal *et al.*, 2006), hyperthermic conditions from fertilisation until after the juvenile stage (Ytteborg *et al.*, 2010b), rearing location combined with low dietary phosphorus (Sullivan *et al.*, 2007b), and vaccination (Berg *et al.*, 2006; Aunsmo *et al.*, 2008; Berg *et al.*, 2012). Other studies demonstrate that vaccination has no effect (Gil-Martens *et al.*, 2010; Grini *et al.*, 2011). Further causative factors are sub-optimal dietary phosphorus during the post-smolt stage (Fjelldal *et al.*, 2009; Smedley *et al.*, 2016), high water temperature during the post-smolt stage (Grini *et al.*, 2011), diets with a high inclusion level of marine feed resources and/or fast growth (Fjelldal *et al.*, 2010), photoperiod (Fjelldal *et al.*, 2005; Wargelius *et al.*, 2009; Fjelldal *et al.*, 2012b), low level of dietary phosphorus during juvenile rearing (Fjelldal *et al.*, 2012a; Fjelldal *et al.*, 2016), low light

intensity (Handeland *et al.*, 2013), heritability (Gjerde *et al.*, 2005), and triploidy (Sutterlin and Collier, 1991; McGeachy *et al.*, 1996; Sadler *et al.*, 2001; Fjelldal and Hansen, 2010; Leclercq *et al.*, 2011; Fraser *et al.*, 2013; Taylor *et al.*, 2013; Taylor *et al.*, 2014; Fjelldal *et al.*, 2016; Smedley *et al.*, 2016). In fact, the aforementioned physiological differences and consequent different requirements deriving from triploidy has led to the hypothesis that triploidy itself is another main causative factor of the occurrence of skeletal anomalies in Atlantic salmon (Benfey, 2015b).

1.4.1 Triploidy and skeletal anomalies

As mentioned above, the higher occurrence of skeletal anomalies in triploids relative to diploids represents the biggest concern for their commercial use. In Atlantic salmon, several authors observed the occurrence of different anomalies in relation to ploidy as shown below in a chronological description. First studies reporting a higher prevalence in triploids of a particular deformation of the lower jaw (identified now as LJD – lower jaw deformity, see below for further details) were Sutterlin *et al.* (1987), Jungalwalla (1991) and McGeachy *et al.* (1996). O'Flynn *et al.* (1997) observed again a prevalence of LJD together with spinal anomalies (scoliosis and lordosis) affecting more triploid individuals. Later, Sadler *et al.* (2001) found again LJD and other types of anomalies (i.e. short opercula and gill filament deformity) with a significant higher prevalence in triploids. Cotter *et al.* (2002), found different types of anomalies (i.e. spinal, caudal, jaw) during the freshwater phase, although at low prevalence, and during the seawater phase found a higher number of triploids with cataracts. Furthermore, they observed spinal anomalies (mainly hump-back type) affecting more triploids before the harvest and the opposite at the harvest. On the other hand, Oppedal *et al.* (2003) observed the same types of anomalies as the previous study (again at low prevalence) but with no differences between ploidies. Fjelldal and Hansen (2010) found higher prevalence of spinal anomalies in triploids. Leclercq *et al.* (2011) reported mostly LJD and spinal anomalies in higher

prevalence in triploids. More recently, different types of anomalies, mainly LJD and spinal anomalies but also cataracts and opercular shortening, were described as affecting more triploid individuals than diploids (Fraser *et al.*, 2013; Taylor *et al.*, 2013; Taylor *et al.*, 2014; Fjelldal *et al.*, 2016; Smedley *et al.*, 2016). In particular, it has been shown that inadequate culture conditions can result in triploids developing a higher prevalence of skeletal anomalies than diploids as demonstrated for higher rearing temperature (Grini *et al.*, 2011), higher incubation temperature (Fraser *et al.*, 2015) and deficiency in dietary P (Fjelldal *et al.*, 2016; Smedley *et al.*, 2016).

These studies highlighted the correlation between triploidy, and interaction between ploidy and other factors, and the occurrence of skeletal anomalies. In fact, the potential physiological differences between ploidies are likely exacerbated by environmental conditions and different nutritional requirements (Benfey, 2015b) impacting skeletal development, mineralisation and the shaping of cartilage and bone through re-modelling processes.

1.4.2 Lower jaw deformity in Atlantic salmon

As shown above, an anomaly commonly reported for farmed Atlantic salmon is lower jaw deformity (LJD). LJD has been described as the downward curvature of the lower jaw involving dentary and glossohyal bones (Bruno, 1990; Hughes, 1992). The lower jaw is an association of different tissues forming a heterogeneous organ (Hughes, 1992). As observed through histological examinations the bone of the lower jaw is composed of compact bone hence it is formed directly by intramembranous ossification around the supporting Meckel's cartilage in Atlantic salmon (Hughes, 1992; Sadler, 2000; Witten and Hall, 2002; Gillis *et al.*, 2006).

LJD has been identified both in freshwater and seawater phases of production in Scotland (Bruno, 1990; Leclercq *et al.*, 2011; Taylor *et al.*, 2013), Ireland (Quigley, 1995), Norway (Oppedal *et al.*, 2003; Fraser *et al.*, 2015; Fjelldal *et al.*, 2016; Smedley *et al.*, 2016),

Canada (Sutterlin *et al.*, 1987; Sutterlin and Collier, 1991; McGeachy *et al.*, 1995; O'Flynn *et al.*, 1997; Benfey, 2001), Chile (Branson and Nieto, 1999; Goicoechea *et al.*, 1999; Rojas *et al.*, 2000; Roberts *et al.*, 2001; Venegas *et al.*, 2003) and Australia (Jungalwalla, 1991; Hughes, 1992; King and Lee, 1993; Lee and King, 1994; Sadler *et al.*, 2001; Lijalad and Powell, 2009). This skeletal anomaly has been relatively common in triploid Atlantic salmon from Tasmania (Jungalwalla, 1991; Hughes, 1992; King and Lee, 1993; Lee and King, 1994; Sadler *et al.*, 2001; Lijalad and Powell, 2009) Canada (Sutterlin *et al.*, 1987), and recently in Scottish stocks (Leclercq *et al.*, 2011; Taylor *et al.*, 2013; Smedley *et al.*, 2016) and at a low prevalence (< 1.5%) in Norwegian stock (Oppedal *et al.*, 2003).

In Tasmania, LJD has been reported to impact up to 30% of commercially produced all-female triploid Atlantic salmon during the seawater phase of the production cycle (Jungalwalla, 1991; Hughes, 1992) and also in lower percentages in freshwater (2% during the fry stage, 7% during the pre-smolt stage) (Sadler *et al.*, 2001). Given that triploid populations have a fundamental role in the annual harvest cycle, this skeletal anomaly results in a considerable loss to salmon farmers (Sadler *et al.*, 2001). In fact, according to Benfey (2001), it is not possible to sell a whole Atlantic salmon with LJD, although it can be sold processed (e.g. steaks and fillets). In addition, jaw anomalies are considered a serious problem affecting fish since they can have deleterious effects principally on feeding and growth but can also lead to inefficient resource use during the production process and further economic losses (e.g. intensive hand grading and wasted feeding) (Bogliione *et al.*, 2013b; Cobcroft and Battaglene, 2013).

There are several components of the Atlantic salmon production cycle with potential to impact on lower jaw structural development, likely including some of the same factors that impact on vertebral development (i.e. dietary P deficiency). There is still uncertainty regarding the fundamental causes of LJD and the mechanisms underlying it. LJD may be caused by

environmental factors and genetic factors (e.g. triploidy) associated with different physiological characteristics or a combination of them. Among the possible causes, deficiency of vitamin C has been reported as a possible cause of LJD by Hughes (1992) and King and Lee (1993), although its role remains unclear. Roberts *et al.* (2001) suggested that LJD can be caused by high respiration rates (causing quick movements of the jaws) due to low levels of dissolved oxygen, in combination with poor mineralisation. Furthermore, LJD prevalence may increase when fish are subjected to higher growth rates as suggested in two recent studies (Taylor *et al.*, 2014; Fraser *et al.*, 2015). Recently, Fjelldal *et al.* (2016) and Smedley *et al.* (2016) showed that triploids have different dietary phosphorus requirements and that P deficiency during the freshwater and seawater phases is linked to the occurrence of LJD in both triploid and diploid Atlantic salmon, with an evidently higher incidence in triploids. In fact, as already mentioned above, the incidence of LJD has been associated mainly with triploids in all studies available potentially implicating a difference in physiological mechanisms originating from the presence of additional genetic material (Jungalwalla, 1991; Hughes, 1992; King and Lee, 1993; Lee and King, 1994; McGeachy *et al.*, 1995; O'Flynn *et al.*, 1997; Benfey, 2001; Sadler *et al.*, 2001; Fjelldal *et al.*, 2011; Leclercq *et al.*, 2011; Fraser *et al.*, 2013; Taylor *et al.*, 2013; Benfey, 2015b). In addition, it has been excluded that LJD may be congenital and sex-dependent as no differences in prevalence between males and females have been found (Taylor *et al.*, 2013).

An additional problem regarding LJD is that the timing of the onset is not clear given that several authors reported the occurrence of LJD during the seawater phase only (Bruno, 1990; Jungalwalla, 1991; Hughes, 1992; King and Lee, 1993; Lee and King, 1994; Quigley, 1995; McGeachy *et al.*, 1996; Leclercq *et al.*, 2011; Taylor *et al.*, 2013; Smedley *et al.*, 2016). Nevertheless, this was probably due to the fact that the anomaly assessment was performed exclusively during that phase and not earlier. Conversely, other authors observed LJD before

the transfer to seawater (Branson and Nieto, 1999; Goicoechea *et al.*, 1999). Sutterlin *et al.* (1987) observed LJD during the freshwater phase (parr stage 5-10 cm) at high prevalence (26 to 51%). O'Flynn *et al.* (1997) found LJD in both freshwater and seawater, but the prevalence and evidence were higher during the latter phase. Sadler *et al.* (2001) observed LJD again during the both phases, in particular at low prevalence during the fry stage (2%), and higher during the pre-smolt stage (7%) and in seawater smolts (14%). Recently, Fjelldal *et al.* (2016) have highlighted that early parr stage (around ~9 g) may be critical for the onset of LJD. In light of the above, the onset of LJD seems to occur at any time in development of the fish although early stages could be more likely the most sensitive period for the onset and/or predisposition to later manifestation of this skeletal anomaly. An additional feature of LJD is that it seems to be progressively more severe, at least in some individuals, with growth (i.e. increase in the downward curvature) (Hughes, 1992) although that remains elusive. Nevertheless, possible exacerbating factors of LJD severity have never been explored.

1.5 Teleosts skeletal system

This last section provides fundamental and comprehensive recent developments in the understanding of Teleost cartilage and bone and the mechanisms leading to their formation and resorption, which are directly applicable to current knowledge of the occurrence of skeletal anomalies and will help the reader to follow the topics addressed in the following chapters.

1.5.1 Skeletal tissues: structure and main types

As in all vertebrates, teleosts present four main categories of mineralised tissue: bone, cartilage, dentine and enamel/enameloid (Witten and Huysseune, 2009; Boglione *et al.*, 2013a; Hall, 2015 page 3). Furthermore, teleosts display several different intermediate skeletal tissue types (Witten and Huysseune, 2009; Boglione *et al.*, 2013a; Witten and Hall, 2015). In the development of mineralised tissues a wide set of cells is involved, the main cell groups are

chondroblasts, chondrocytes, osteoblasts, bone lining cells, osteocytes, osteoclasts, odontoblasts, ameloblasts (Boglione *et al.*, 2013a; Hall, 2015 page 3).

Bone is a supporting skeletal tissue originating from mesenchyme (Boglione *et al.*, 2013a; Hall, 2015 page 5). Inside the bone, calcium and phosphorus are stored and mineral homeostasis takes place (Hall, 2015 page 5). The main component of bones is the organic matrix, consisting of type I collagen, crystals of hydroxyapatite (deriving from calcium phosphate) and non-collagenous proteins such as osteocalcin, osteopontin and osteonectin (Hall, 2015 page 5). Bones contain blood vessels that enable gas exchange in the tissue (review by Moss, 1961; Boglione *et al.*, 2013a ; Hall, 2015 page 5). Haematopoietic tissue is not present in fish bone marrow (as located in the head kidney) and is usually replaced by adipose tissue (Zapata *et al.*, 2006; Witten and Huysseune, 2009). The cells that are present in bones are osteoblasts, osteocytes, osteoclasts and bone lining cells (Boglione *et al.*, 2013a; Hall, 2015 page 5). As observed by Moss (1961), the presence of osteocytes in bone distinguishes the cellular bone from the acellular bone. Basal teleosts, such as Atlantic salmon, have cellular bone. Conversely, more advanced teleosts have acellular bone (Moss, 1961; Witten *et al.*, 2004). The terms basal and advanced refer to phylogenetic groups which are more or less primitive, respectively. In this case, advanced teleosts had their origin approximately 100 million years after basal teleosts (Witten and Huysseune, 2009). Bone growth in fish occurs in two phases, the first in which the bone is initially a woven bone and the second in which the woven bone becomes parallel-fibred and lamellar bone (Boglione *et al.*, 2013a). As reviewed by Boglione *et al.* (2013a) there are three principal mechanisms leading to bone formation in teleosts. Endochondral ossification in which a cartilaginous template, formed by chondrocytes differentiating from mesenchymal stem cells (Erlebacher *et al.*, 1995), is gradually replaced by bone (Boglione *et al.*, 2013a). Perichondral ossification, the most common in fish and especially for the fin endoskeleton, occurs by the transformation of cells initially belonging to

perichondrium into cells of the periosteum (osteoblast secreting bone matrix) (Boglione *et al.*, 2013a). The last mechanism is intramembranous ossification in which a cartilaginous template is not present and bone is formed directly by osteoblasts differentiating from mesenchymal stem cells (Boglione *et al.*, 2013a) (Fig. 1.3). Another important process in skeletal development is bone resorption and remodelling. During bone resorption, bone is removed by bone resorbing cells, called osteoclasts (Fig. 1.4), and new bone is formed by the above mentioned osteoblasts and osteocytes (bone forming cells) with osteoblasts turning into osteocytes as the last stage (Boglione *et al.*, 2013a). This process underlies mainly allometric growth but is necessary also for bone repair (Witten and Huysseune, 2009), as adaptation to mechanical load (Hall and Witten, 2007), for tooth replacement and for removing temporary skeletal elements as reviewed by Boglione *et al.* (2013a). Fish have two types of osteoclasts, small mononucleated that are found in advanced teleosts, and large multinucleated in basal teleosts (e.g. Atlantic salmon) (Witten and Huysseune, 2009). Basal teleosts also possess mononucleated osteoclasts, but only during the early stages of development when they have acellular bone, while in advanced teleosts mononucleated osteoclasts are preeminent throughout the development (Sire *et al.*, 1990; Witten and Huysseune, 2009).

Cartilage is another important skeletal tissue. Cartilage can or cannot ossify during development and several types of cartilage have been described in fish (Witten and Huysseune, 2009; Witten *et al.*, 2010; Boglione *et al.*, 2013a; Hall, 2015 page 5-11). The main function of cartilage is support, as is the main function for bone (Witten and Huysseune, 2009; Hall, 2015 page 5-11). Nevertheless, cartilage has also a key role as articular tissue in vertebrates (Witten and Huysseune, 2009; Hall, 2015 page 5-11). Different from bone, cartilage does not contain blood vessels (Witten *et al.*, 2010; Hall, 2015 page 5-11). The extracellular matrix (ECM) of cartilage is mainly composed of proteoglycans and chondroitin sulphates and, although other collagens are present, type II collagen is the most abundant protein and is considered specific

for the tissue (Witten *et al.*, 2010; Hall, 2015 page 5-11). The cells responsible for cartilage formation are chondroblasts and chondrocytes (Witten and Huysseune, 2009; Witten *et al.*, 2010; Hall, 2015 page 5-11). Cartilage can also undergo resorption by resorbing cells called chondroclasts (Witten and Huysseune, 2009; Witten *et al.*, 2010; Hall, 2015 page 5-11).

1.5.2 Cellular and molecular mechanisms underlying bone and cartilage formation and resorption

The synchronized interaction of osteoblasts and osteoclasts is fundamental in order to avoid the onset of skeletal diseases and malformations (Witten and Huysseune, 2009). Several factors regulate the differentiation of osteoblasts leading to bone formation (Ytteborg *et al.*, 2012) (Fig. 1.3). Regarding the osteoblasts, four principal phases of development have been described: lineage commitment, proliferation, ECM production and mineralisation (Karsenty, 2008; Karsenty *et al.*, 2009; Javed *et al.*, 2010) (Fig. 1.3). The expression of several genes is required during the process of differentiation (Karsenty, 2008; Karsenty *et al.*, 2009; Javed *et al.*, 2010). During the commitment phase several key transcription factors play an important role such as runt-related transcription factor 2 (Runx2), osteoblast-specific transcription factor (Osterix), Sox9, and morphogens such as transforming growth factor β /bone morphogenetic proteins (TGF β /BMPs) and fibroblast growth factors (FGFs) (Karsenty, 2008; Javed *et al.*, 2010). In addition, other molecules, including Vitamin D3, glucocorticoids, parathyroid hormone (PTH), and estrogen influence this stage. In the second phase, proliferation, the expression of genes that are mostly related to matrix formation such as type I collagen (produced by the gene *Colla1*), fibronectin and growth factors such as BMP2/TGF β starts (Karsenty, 2008; Karsenty *et al.*, 2009; Javed *et al.*, 2010). During the third phase, production of matrix, synthesis and maturation of ECM occurs, with the expression of alkaline phosphatase (ALP) an early marker of osteogenesis, and the production of non-collagenous ECM proteins such as osteopontin, osteonectin, bone sialoprotein, and osteocalcin that enhance the development, together with

collagen, of the ECM (Karsenty, 2008; Karsenty *et al.*, 2009; Javed *et al.*, 2010). In the last phase, mineralisation, the deposition of minerals (mainly Ca and P) in the ECM takes place, highlighted by the expression of genes producing bone sialoprotein, osteocalcin, and osteopontin that are considered markers of mature osteoblasts (Karsenty, 2008; Karsenty *et al.*, 2009; Javed *et al.*, 2010). The beginning of this phase is characterised by the down regulation of matrix maturing proteins and the simultaneous expression of genes related to the development and deposition of hydroxyapatite crystals (Karsenty, 2008; Karsenty *et al.*, 2009; Javed *et al.*, 2010).

In addition, it is appropriate to introduce not only the mechanisms strictly related to direct bone formation but also those underlying cartilage formation, or chondrogenesis, since it represents the preceding step of bone formation in some cases. Chondroblasts and chondrocytes (cartilage forming cells) derive from the same cellular precursors as osteoblasts, and these precursors can differentiate into one or the other under the regulation of transcription factors such as Sox9, Runx2 and Osterix (Karsenty *et al.*, 2009; Javed *et al.*, 2010). After the differentiation there is a phase of proliferation in which parathyroid hormone related protein (PTHrP) and Indian hedgehog (Ihh) seem to contribute to maintain the cells in a proliferative condition (Ytteborg *et al.*, 2012). During this phase, proliferating chondrocytes produce an ECM made of type II collagen (produced by the gene *Col2a1*), (Karsenty *et al.*, 2009). The last phase is the maturation of chondrocytes that become hypertrophic. This phase is under control of two main transcription factors, myocyte enhancer factor 2C (Mef2c) and Runx2, and hypertrophic chondrocytes that produce type X collagen (Karsenty *et al.*, 2009).

As mentioned above, the principal cells involved in bone resorption are osteoclasts (Fig. 1.4). In mammals, these cells originate from haematopoietic tissue in the bone marrow (Witten and Huyseune, 2009). In teleosts, although this tissue is located in the head kidney, the origin of the osteoclasts remains unknown (Witten and Huyseune, 2009). The role of the osteoblasts

is fundamental for the differentiation and activation of osteoclasts (Fig. 1.4) (Boyle *et al.*, 2003; Karsenty *et al.*, 2009). Osteoclast formation is mediated by the osteoblasts-derived cytokines: macrophage colony stimulating factor (M-CSF) and the receptor activator of nuclear factor κ B ligand (RANKL) that are directly involved in osteoclastogenesis. M-CSF activates its receptor colony stimulating factor (C-FMS) in the osteoclast precursor that controls osteoclasts production, differentiation and function (Karsenty *et al.*, 2009; Javed *et al.*, 2010). RANKL belongs to the tumor necrosis factor (TNF) family. It interacts with M-CSF, allowing the activation of receptor activator of nuclear factor κ B (RANK) and osteoclast fusion and differentiation (Boyle *et al.*, 2003; Javed *et al.*, 2010). In addition, another cytokine belonging to the TNF family, is produced by osteoblasts, named osteoprotegerin (OPG), which is also involved in osteoclastogenesis (Boyle *et al.*, 2003). OPG regulates the production of osteoclasts, generally blocking it, and its interaction with RANKL controls the bone resorption activation or deactivation by modifying the state of RANK on osteoclasts (Boyle *et al.*, 2003). The described mechanism, involving the interaction between different types of molecules, leads to the maturation of the osteoclasts through the expression of genes of the osteoclast lineage and the production of two lysosomal enzymes such as tartrate-resistant acid phosphatase (TRAP) and cathepsin K (CATK), and in addition calcitonin receptor (Boyle *et al.*, 2003). Also matrix metalloproteinases (MMP) seem to be involved in bone resorption (Delaissé *et al.*, 2003; Witten and Huysseune, 2009; Ytteborg *et al.*, 2010a; Fjellidal *et al.*, 2012b; Ytteborg *et al.*, 2012). As reviewed by Witten and Huysseune (2009) and Boyle *et al.* (2003), after the junction with the bone surface, the action of a vacuolar proton pump (H-ATPase) acidifies the extracellular environment aiding dissolution of bone and the function of lysosomal enzymes (TRAP and CATK) that breaks down ECM forming resorption pits (Howship's lacunae).

The mechanisms underlying bone and cartilage formation and resorption described above are based on mammalian models. In teleosts the same mechanisms remain to be

understood. Nevertheless, specific literature can be used to partly understand differences and similarities between teleosts and mammals (e.g. Spoorendonk *et al.*, 2010; Ytteborg *et al.*, 2010a; Apschner *et al.*, 2011).

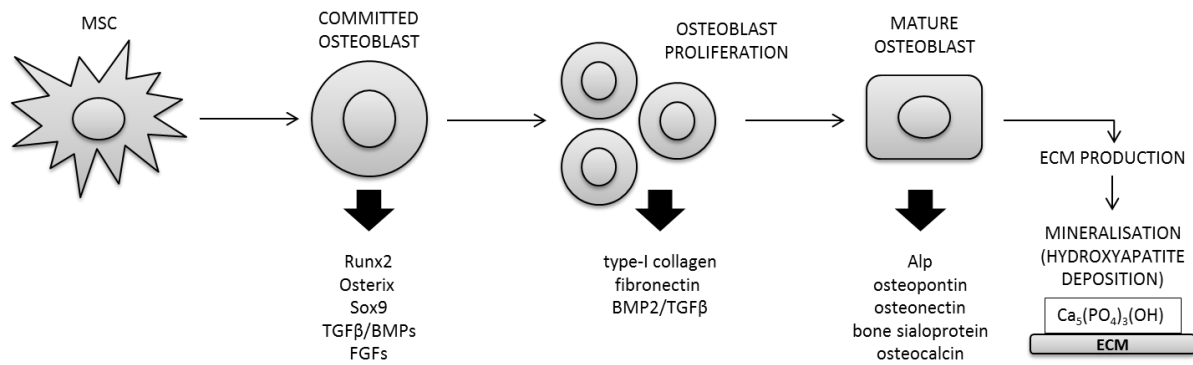


Fig. 1.3. Osteoblastogenesis. From the differentiation of a bone forming cell (osteoblast) to the mineralisation of the extracellular matrix (bone formation) and the different factors involved. Horizontal arrows indicate the passages between different cellular phases, vertical arrows indicate some of the fundamental genes (biomarkers) expressed during each cellular phase. (Alp = alkaline phosphatase, BMP = bone morphogenetic protein, ECM = extracellular matrix, FGF = fibroblast growth factor, MSC = mesenchymal stem cell, TGF = transforming growth factor). The figure is inspired by the several illustrations produced on the topic without referring to any in particular.

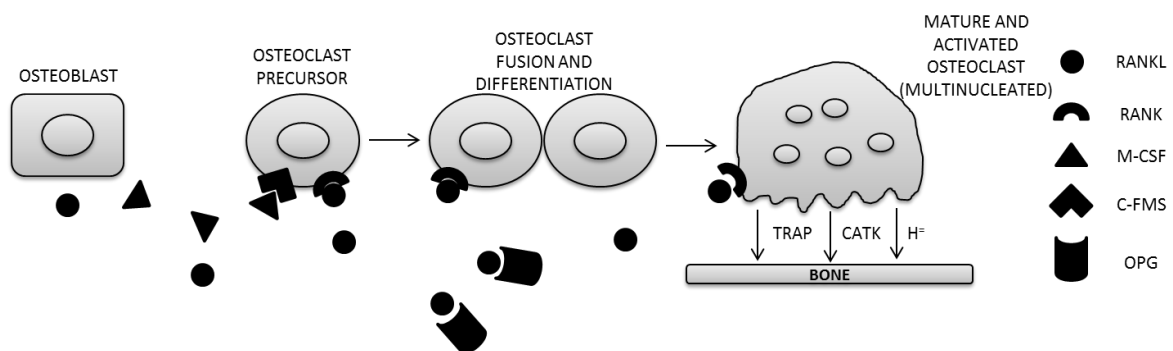


Fig. 1.4. Osteoclastogenesis. From the differentiation of a bone resorbing cell to the resorption of bone and the different factors involved. Horizontal arrows indicate the passages between different cellular phases, vertical arrows indicate some of the fundamental genes expressed (biomarkers), black shapes represent at the same time which genes are expressed during the cellular phases and the interaction between products and their receptors leading to differentiation and activation or inactivation of a mature osteoclast. (C-FMS = receptor colony stimulating factor, CATK = cathepsin, K RANKL = receptor activator of nuclear factor κ B ligand, M-CSF = macrophage colony stimulating factor, OPG = osteoprotegerin, RANK = receptor activator of nuclear factor κ B, TRAP = tartrate-resistant acid phosphatase). The figure is inspired by the several illustrations produced on the topic without referring to any in particular.

1.6 Aims of the research

The general intent of this project was to contribute to Atlantic salmon industry in order to understand, and direct research toward a possible solution to, the problem of skeletal anomalies in Atlantic salmon in order to enhance productivity and fish welfare both locally and globally. This PhD research thesis was focused on exploring the occurrence of several skeletal anomalies in farmed triploid (and diploid) Atlantic salmon in freshwater that represent a critical developmental phase. In particular, the main aim was to improve our factorial knowledge of lower jaw deformity (LJD) occurrence and understand the molecular mechanism underlying this anomaly. In the following paragraphs, the outline of the research chapters will be presented in order to show to the reader the logical and consecutive steps taken to fulfil the predetermined aims.

The second chapter describes the first experimental assessment undertaken during this research. Diploids and triploids were maintained in controlled conditions for nine months (from incubation to 60 g) and repeatedly screened (visually and by using x-ray) for the occurrence of several categories of skeletal anomalies (i.e. lower jaw anomalies, opercular shortening, spinal anomalies) and the effect of standard and elevated temperature regimes (14 and 18 °C) in the pre-smolt stage was tested. The main focus of the first experiment was to confirm LJD occurrence to be mainly in triploids, understand the time of onset and test the effect of temperature on its prevalence.

The third chapter describes an experiment developed following the results of the second chapter, on LJD developmental patterns in triploids and LJD concurrence with another lower jaw anomaly affecting Atlantic salmon, shortened lower jaw (SJ). For this experiment only triploid individuals (~12 g) were used, they were selected at a commercial hatchery, allocated into two separate groups according to their lower jaw condition (normal or affected by SJ), grown out in controlled conditions and assessed (visually and by image analysis) over three

months for concurrence of SJ and LJD. The main aim of the experiment was to verify concurrence of lower jaw skeletal anomalies in the two groups and understand through their developmental patterns independency or dependency of the conditions. Furthermore, effect of lower jaw anomalies on growth and recovery from opercular shortening was tested.

The fourth chapter describes the first molecular analysis targeting fish affected by LJD. Two independent sample sets of triploid Atlantic salmon, experiment-sourced (60 g) (from the experiment described in the second chapter) and commercially produced (100 g) displaying LJD and with a normal lower jaw were used to delineate multigenicity of the condition. The main aim was to investigate, by using transcriptome analysis, real-time qPCR and *in-situ* hybridization, molecular mechanisms underlying the condition and to detect possible candidate genes providing clues on tissue/s responsible for the trait. The use of two independent sample sets at different developmental stages helped to strengthen the outcome of the analyses and verify differential gene expression consistency. The effect of temperature on gene regulation was also tested.

CHAPTER 2: Skeletal anomaly assessment in diploid and triploid juvenile Atlantic salmon (*Salmo salar* L.) and the effect of temperature in freshwater

Gianluca Amoroso^a, Mark B. Adams^a, Tomer Ventura^b, Chris G. Carter^a, Jennifer M. Cobcroft^{a,b}

^a Institute for Marine and Antarctic Studies (IMAS), University of Tasmania

^b Genecology Research Centre, School of Science and Engineering, University of the Sunshine Coast

Keywords: aquaculture, Atlantic salmon, lower jaw deformity, opercular shortening, triploid, vertebral deformities.

This manuscript has been published in *Journal of Fish Diseases* (2016) 39(4) 449-466

2.1 Abstract

Triploid Atlantic salmon tend to develop a higher prevalence of skeletal anomalies. This tendency may be exacerbated by an inadequate rearing temperature. Early juvenile all-female diploid and triploid Atlantic salmon were screened for skeletal anomalies in consecutive experiments to include two size ranges: the first tested the effect of ploidy (0.2–8 g) and the second the effect of ploidy, temperature (14 °C and 18 °C) and their interaction (8–60 g). The first experiment showed that ploidy had no effect on skeletal anomaly prevalence. A high prevalence of opercular shortening was observed (average prevalence in both ploidies 85.8%) and short lower jaws were common (highest prevalence observed 11.3%). In the second experiment, ploidy, but not temperature, affected the prevalence of short lower jaw (diploids > triploids) and lower jaw deformity (triploids > diploids, highest prevalence observed 11.1% triploids and 2.7% diploids) with a trend indicating a possible developmental link between the two jaw anomalies in triploids. A radiological assessment ($n = 240$ individuals) showed that at both temperatures triploids had a significantly ($P < 0.05$) lower number of vertebrae and higher prevalence of deformed individuals. These findings (second experiment) suggest ploidy was more influential than temperature in this study.

2.2 Introduction

The commercial use of triploid Atlantic salmon has been explored for many years (Benfey and Sutterlin, 1984b; Sutterlin *et al.*, 1987; Johnstone *et al.*, 1991; Jungalwalla, 1991; Carter *et al.*, 1994) and interest is now high due to their possible use to mitigate the effect of escapes and consequent interbreeding with wild populations. Nevertheless, triploids have a higher predisposition to develop skeletal anomalies (Leclercq *et al.*, 2011; Fraser *et al.*, 2013; Taylor *et al.*, 2013; Fraser *et al.*, 2015; Fjellidal *et al.*, 2016). Triploid Atlantic salmon have been produced on a commercial basis exclusively in Australia for almost 20 years, the aim is to ensure appropriately sized harvest fish all year round (Sadler, 2000; Benfey, 2009). In

Tasmania, Australia, three different population types of Atlantic salmon are produced commercially: mixed sex diploids, all-female diploids and all-female triploids (Sadler, 2000). Triploid Atlantic salmon have similar or better survival and growth rates than diploids, a desirable factor to shorten production cycles and supply bigger smolts for on-growing, and have improved flesh quality at harvest since they are not subject to the detrimental effect of maturation (Benfey, 1999; Sadler *et al.*, 2000b; Taylor *et al.*, 2013). Nevertheless, the higher prevalence of skeletal anomalies reported in triploids, mainly affecting the vertebral column and the lower jaw, has limited their use for commercial production (Benfey, 2001; Sadler *et al.*, 2001; Fraser *et al.*, 2012a; Taylor *et al.*, 2013; Fjelldal *et al.*, 2016). The prevalence and the frequency of each skeletal anomaly type vary between countries, studies and populations (as reviewed by Fraser *et al.*, 2012a). Skeletal anomalies represent a global issue in cultured fish, not only in triploids, having deleterious effects on welfare and growth and leading to significant economic losses due to poor quality of the juveniles and adults produced and expensive grading processes (Boglione *et al.*, 2013a; Cobcroft and Battaglene, 2013). Given the current and potential importance of triploids, predisposing factors and causation of skeletal anomalies in Atlantic salmon require elucidation. Skeletal anomalies are caused by several factors, most important are environmental (e.g. temperature, photoperiod, water quality, rearing methods), nutritional (e.g. deficiency of minerals and vitamins) or genetic (polyploidy, selective breeding, inbreeding) (Lall and Lewis-McCrea, 2007; Fjelldal *et al.*, 2012c; Boglione *et al.*, 2013b). Nevertheless, in Atlantic salmon, high temperature and dietary phosphorus (P) deficiency, applied during both incubation and rearing, are the principal factors that negatively impact skeletal development (Fjelldal *et al.*, 2012c; Fjelldal *et al.*, 2016). In particular, temperature, that has a well-known effect on growth, is manipulated during the production cycle to achieve higher growth rates (Fjelldal *et al.*, 2012c). Following the findings of several studies on triploid Atlantic salmon showing that triploids develop higher prevalence of skeletal

anomalies than diploids when subjected to the same rearing conditions (inadequate or not), triploidy can now be considered as an additional important causative factor although the mechanisms underlying this issue still need to be clarified (Benfey, 2001; Sadler *et al.*, 2001; Leclercq *et al.*, 2011; Taylor *et al.*, 2011; Fraser *et al.*, 2013; Taylor *et al.*, 2013; Fjelldal *et al.*, 2016). The negative interaction between ploidy, accelerated growth, deficient dietary P and the consequent imbalance in the natural time for bone mineralisation represents an additional obstacle to reduce skeletal anomalies in commercial operations. Furthermore, many differences in physiology between diploids and triploids remain unknown as well as the effect of inadequate temperature regimes on triploids at different developmental stages, requiring further research. Previous studies tested the effect of high temperatures on diploids (Ytteborg *et al.*, 2010b) or both ploidies (Grini *et al.*, 2011; Fraser *et al.*, 2013; Fraser *et al.*, 2014a) and applying, in some cases, high temperature regimes during the incubation only and focusing mostly on vertebral deformities occurrence. The aim of this study was to assess the effect of ploidy and temperature on the prevalence of several categories of skeletal anomalies in juvenile diploid and triploid Atlantic salmon. We focussed the investigation on two factors: the effect of ploidy on the prevalence of skeletal anomalies in particular affecting the lower jaw (specifically, lower jaw deformity [LJD] and short lower jaw [SJ]) and the effect of temperature (14 °C and 18 °C), ploidy and their interaction on the prevalence of all skeletal anomalies, including vertebral deformities, in both diploids and triploids during a critical developmental phase, the parr/pre-smolt stage.

2.3 Materials and methods

2.3.1 Fish stock

Atlantic salmon eggs were provided by Petuna Seafoods hatchery in Cressy, Tasmania in May 2013. About 37 000 eggs were pooled from six females and were fertilised with the milt of two

neomales (sex-reversed genotypic females) (Johnstone *et al.*, 1991; Jungalwalla, 1991), resulting in all-female embryos. The fertilised eggs were divided into two batches. 30 min after fertilisation, one batch of eggs was subjected to a pressure shock to induce triploidy for 4 min at 9500 psi (655 Bar) in water at 10 °C, followed by 1 min of pressure release (Johnstone *et al.*, 1991; Jungalwalla, 1991). The two egg batches, triploids (3N) and diploids (2N), were placed separately in trays and transported (1 h) to the IMAS Launceston Aquaculture Centre at the University of Tasmania. All procedures were carried out with the approval of the University of Tasmania Animal Ethics Committee (approval number A0013044).

2.3.2 Embryo incubation

Embryo incubation occurred in two troughs (120 L capacity) each containing four upwelling hatching trays (44.5 × 39.5 × 14.5 cm). The fertilised eggs were gently placed into the eight incubation trays ($n = 4$ per ploidy) allocating them equally by volume using a 250 mL beaker. Each tray contained alternate diploid or triploid eggs and the two troughs had opposite disposition of diploids and triploids in the trays. The troughs were continuously exchanged with fresh water (120 L per hour) from a re-circulating system consisting of a reservoir tank (4000 L) exchanged at 10% daily with mains water de-chlorinated with appropriate amounts of sodium thiosulphate, a biofilter, heat-chill and UV disinfection unit. The fertilised eggs were kept in the dark until hatching. Water quality parameters including pH, ammonia (NH₃), nitrite (NO₂), nitrate (NO₃) (Freshwater Master Test Kit, API Aquarium Pharmaceuticals), dissolved oxygen (O₂), (Orion Star A223 RDO/DO meter, Thermo scientific) and chlorine (Cl₂) (DPD Total Chlorine Reagent, HACH PermaChem Reagents) were measured weekly. The pH was maintained at 7.2 ± 0.3 (values for water parameters are mean \pm SD, here and throughout) and dissolved oxygen at 100% saturation throughout incubation, the other parameters were within safe operating limits (NH₃ 0.12 ± 0.12 ppm, NO₂ 0.01 ± 0.05 ppm and NO₃ 1.5 ± 2.3 ppm) (Eddy and Williams, 1987). Water temperature was 7.8 ± 0.2 °C throughout incubation,

recorded every 15 min with waterproof data loggers (HOBO Pendant, Onset Computer Corporation) (two per trough, placed at the inflow and at the outflow). Egg development rate was described using degree days ($^{\circ}\text{C days}$ – cumulative average daily temperature). Fertilised eggs were ‘eyed’ at 271 $^{\circ}$ days (35 days post fertilisation – dpf), and hatching started at 426 $^{\circ}$ days and was completed at 488 $^{\circ}$ days (55 and 63 dpf, respectively). After the ‘eyed’ period (at 310 $^{\circ}$ days, 40 dpf) the embryos were shocked to remove the unfertilised eggs (trays were removed and replaced in the troughs subjecting embryos to a sufficient up-flow of water to break the shell of unfertilised eggs) (Fjelldal *et al.*, 2016). Dead (white) eggs were removed (by siphon) and counted on a daily basis. Mortalities are reported as a percentage of the total dead individuals on total stocked from here on.

2.3.3 Viability rate assessment

A viability rate assessment was performed at 162 $^{\circ}$ days (21 dpf) to evaluate the percentage of eggs successfully developing. According to the standard industry procedure, 80 eggs per ploidy were sampled and placed in 7% acetic acid for 5 min. Viability was confirmed by notochord development, indicated by a straight white line, and the proportion of viable embryos was determined.

2.3.4 Experiment 1: early rearing (alevin – fry)

A total of 7800 alevins ($n = 3900$ per ploidy) were moved at 589 $^{\circ}$ days and allocated into three re-circulating systems for first feeding and early rearing. Each system consisted of one tank (4000 L) supplied with de-chlorinated mains water (as described above) and re-circulated through a biofilter, heat-chill unit and UV disinfection unit. Each tank contained four replicate crates (32 L – $42 \times 32 \times 29.5$ cm; $n = 2$ per ploidy) with alternate diploid or triploid fish ($n = 650$ per crate). Stocking density during use of crates was 4.1 kg m^{-3} at the beginning and 50 kg m^{-3} at the end (stocking density was calculated considering individuals removed at each sampling event here and throughout). At 1988 $^{\circ}$ days the crates were replaced by floating mesh

cages (320 L – 50 × 50 × 92 cm). Stocking density during use of mesh cages was 4.8 kg m⁻³ at the beginning and 12.8 kg m⁻³ at the end. The water exchange was set at 20% daily in the tanks and 100% every 20 min in the crates. The water quality parameters during the experiment were pH 7.0 ± 0.2 and dissolved oxygen 94.3 ± 3.5% saturation, the other parameters were within safe operating limits (NH₃ 0.08 ± 0.12 ppm, NO₂ 0.14 ± 0.31 ppm and NO₃ 9.5 ± 8.1 ppm) (Eddy and Williams, 1987). Water temperature was recorded every 15 min with waterproof data loggers (HOBO Pendant, Onset Computer Corporation) (one per tank). Water temperature was maintained at 8.4 ± 0.2 °C until one week prior to first feeding (765° days), when the temperature was gradually increased to 10 °C at first feeding. One week after first feeding temperature was increased again to 11 °C and then gradually to 14 °C over the course of two weeks, and was maintained at 14.2 ± 0.1 °C until the end of the experiment (2499° days). Fish were exposed to a 24-h light photoperiod throughout the rearing phase by using a spotlight mounted above each tank equipped with Philips 300 W halogen globe and with an intensity of 500 lux at the water surface. All the fish were fed with Nutra XP and RC diets (Skretting) depending on the stage of development in accordance with industry recommendations. Feeds were delivered to satiation at a feeding rate of eight times per day with Nutra XP and three times per day with Nutra RC. At 2272° days fish were bath vaccinated for *Yersinia ruckeri* in a 10% vaccine solution for 45 sec (YERSINIAVAC-B, Intervet). Dead fish were removed daily by siphon or dip-net depending on the size of fish and all mortalities were recorded.

2.3.5 Experiment 1: sampling

Fish were sampled ($n = 120$ per ploidy per sampling event) at weekly intervals, four times before first feeding (844° days), then at monthly intervals for four months until the end of the first experiment (2499° days). Fish were killed by anaesthetic overdose (AQUI-S: 50 mg L⁻¹). Body weight, total length (TL) and fork length (FL; measured once the caudal fin was fully developed) were recorded starting from the second sampling event. Specific growth rate (SGR)

was calculated as $SGR (\% \text{ day}^{-1}) = 100 \times (\ln(W2/W1)) \times d^{-1}$, where $W1$ and $W2$ are the initial and final weights (g) and d the number of days. A visual assessment of skeletal anomalies, performed according to the Tasmanian Salmonid Growers Association epidemiological survey of deformity and descriptions in the literature, was undertaken during the last two sampling events of the first experiment at 1973° days (when fish had attained a mean length (FL) of > 65 mm and weight > 3 g) and at 2400° days (~8 g) on all the individuals sampled ($n = 120$ per ploidy).

2.3.6 Experiment 2: juvenile rearing (parr/pre-smolt) at two temperatures

A total of 2760 fish ($n = 1380$ per ploidy) approximately 8 g body weight were allocated at 2514° days into a new system to assess the effect of temperature on skeletal anomalies over a 2-month period. The experiment had a fully orthogonal design, with two levels in each of two factors; ploidy (diploid, triploid) and temperature (14 °C and 18 °C defined as Standard and Elevated treatment, respectively, from now on). Two re-circulating systems, one at 14 °C and the other at 18 °C, were housed in a temperature-controlled room. The systems were supplied with dechlorinated mains water (as described above), each with an independent swirl separator, particulate filter, biofilter, UV disinfection unit and heat-chill unit. Each system had six cylindro-conical tanks (250 L) ($n = 3$ per ploidy) with randomly allocated diploid or triploid fish ($n = 230$ per tank). Stocking density was 7.4 kg m^{-3} at the beginning for both the treatments and 17.6 kg m^{-3} for standard treatment and 9.1 kg m^{-3} for elevated treatment at the end. Freshwater was exchanged at 80% daily for each system and at 100% every 30 min in each tank. The water quality parameters during the experiment were: pH 7.1 ± 0.1 for the standard treatment and 7.1 ± 0.2 for the elevated treatment, dissolved oxygen saturation $84.3 \pm 1.9\%$ for standard treatment and $85.4 \pm 2.0\%$ for elevated treatment throughout the assessment. Other parameters were, on average, within safe operating limits (NH₃ 1.1 ± 1.5 ppm, NO₂ 0.3 ± 0.5 ppm and NO₃ 4.5 ± 3.9 ppm) (Eddy and Williams, 1987). Water temperature was recorded

every 15 min with waterproof data loggers (one each system placed in the biofilter). The temperature in the standard treatment was maintained at 14.2 ± 0.1 °C. In the elevated treatment, temperature was set at 15 °C at the beginning and gradually increased to 18 °C during the first week following the transfer and then maintained at 18.2 ± 0.0 °C until the end of the experiment. Fish were exposed to 24-h light photoperiod throughout the assessment and the experiment room was illuminated by Philips LIFEMAX TLD tubular fluorescent lamps 36 W/840 cool white and intensity of at 150 lux at water surface. All the fish were fed with Nutra RC diet (Skretting) in accordance with industry recommendations, feed was delivered to satiation at a feeding rate of three times per day (09:00, 13:00 and 17:00), and in the second half of experimental period, feed was delivered seven times over a 24-h period through supplementing the original three feeds with automatic feeders four times per day (20:00, 00:00, 03:00, 06:00) to maintain growth rate as close as possible to the industry rate. Dead fish were removed daily by using a dip-net and mortality was recorded.

2.3.7 Ploidy determination

The efficiency of triploid induction and the ploidy status were determined by erythrocyte nuclear length measurements (Benfey *et al.*, 1984) of 240 fish ($n = 120$ per ploidy). A protocol modified from Taylor *et al.* (2011) was used. A drop of blood was collected by cutting the caudal peduncle of killed fish (~2740° and 2980° days), placed on a separate glass slide for each fish and smeared. The slides were air-dried and immediately placed into haematoxylin for 1 min. Photos of the blood smear preparations were taken and the nuclear length measured at 400× magnification using image capture software ImageJ (Schneider *et al.*, 2012). For each individual, five randomly chosen nuclei were measured. The length of nuclei was significantly greater in triploids than diploids ($F_{1238} = 2022.9$, $P < 0.001$; 2N, 6.35 ± 0.03 µm; 3N, 8.85 ± 0.05 µm – mean \pm SEM). Triploid induction was confirmed successful at 100% of the triploid individuals analysed and no triploids were identified in the diploid group.

2.3.8 Experiment 2: sampling

Sampling was standardized by fish development indicated by age in °C days. In the elevated treatment, sampling occurred every ~13 days on five occasions. In the standard treatment, sampling was performed when the fish attained approximately the same degree day age as fish sampled from the elevated treatment, on four occasions before 3434° days (as showed in Table 2.1). Fish were killed as described previously ($n = 126$ per ploidy per temperature in first sampling event and $n = 150$ per ploidy per temperature in consecutive sampling events). Body weight and fork length (FL) were recorded and SGR was calculated as previously described. Fulton's condition factor (K) was calculated as $K = \text{weight} \times 100/\text{length}^3$. A visual assessment of skeletal anomalies, performed as previously described, was undertaken at each sampling event on all individuals sampled ($n = 688$ diploids and $n = 668$ triploids in the elevated treatment and $n = 576$ per ploidy in the standard treatment).

Table 2.1. Sampling events and the equivalent age in °C days (cumulative average daily temperature) of diploid and triploid Atlantic salmon parr/pre-smolts kept in different temperature treatments (Standard 14 °C and Elevated 18 °C) sampled throughout the experiment. The sampling events for the two treatments were performed at the same age (° days) in order to compare same stages of development.

	Temperature treatment			
	Standard 14 °C		Elevated 18 °C	
Sampling	Fish age (° days)	Days from start	Fish age (° days)	Days from start
1	2741	17	2740	14
2	2982	34	2976	27
3	3207	50	3212	40
4	3434	66	3448	53
5	n/a	n/a	3630	63

n/a = not applicable

2.3.9 Identification of skeletal anomalies

All the fish examined for skeletal anomalies were classified as displaying (≥ 1 anomaly type) or not displaying skeletal anomalies and the following categories were used for classification: SJ (tip of lower jaw shorter than the tip of the upper jaw with the mouth closed), lower jaw (dentary and glossohyal) curved downwards also known as LJD (Bruno, 1990; Hughes, 1992), misaligned lower jaw (MA) (not aligned on the longitudinal plane) (Sadler *et al.*, 2001), opercular shortening on the right side (SOR), left side (SOL) or both sides (SOB) (different degrees of shortened operculum/a leaving the gill filaments exposed) (Sadler *et al.*, 2001), spinal anomalies (Sp DEF) (lordosis, kyphosis, scoliosis or externally visible vertebral deformities) (Witten *et al.*, 2009) and fish without anomalies (Normal). The severity of anomalies was not classified.

2.3.10 Radiological assessment

The analysis of vertebral deformities was carried out on fish preserved at -20°C ($n = 240$, 60 per ploidy per temperature treatment) weighing approximately 60 g from the last sampling on both treatments according to the same number of days spent in the treatment (fourth sampling in standard and fifth in elevated treatment) (Table 2.1). Fish were x-rayed using an ECORAY Orange portable x-ray unit (Orange model 1040HF, ECORAY) exposing the plate for 50 mAs and 40 kV at a distance of 70 cm. Images were obtained using a computed radiography system CARESTREAM Vita CR Systems (Carestream Health, Inc.), converted from DICOM to TIFF files and analysed by Image-J (Schneider *et al.*, 2012). The total number of vertebrae in the column was reported counting as distinct vertebral bodies the vertebrae involved in fusions. Fish were considered deformed when ≥ 1 deformed vertebra were observed. Vertebral compressions and fusion were identified, as described by (Witten *et al.*, 2009), but without distinction of types, and the number of affected vertebrae was counted in different vertebral regions. As described by Kacem *et al.* (1998), four main regions (R) were distinguished in the

vertebral column to verify the most affected areas: R1 (cranial trunk) comprising vertebrae (V) V1–V8; R2 (caudal trunk) comprising vertebrae V9–V30; R3 (tail) comprising V31–V49; and R4 (tail fin) comprising V50–V60.

2.3.11 Statistical analysis

One-way ANOVA was used during incubation and the first experiment to investigate the effect of ploidy on mortality, growth (weight, length and SGR) and prevalence of skeletal anomalies and tank was used as random effect. Two-way ANOVA was used during the second experiment to investigate the effects of temperature, ploidy and their interaction on mortality, growth (weight, length, SGR, K) and prevalence of skeletal anomalies and tank was used as random effect. During the second experiment, one-way ANOVA was used to investigate the effect of ploidy within each temperature treatment (elevated and standard considered separately due to standardization per age and the consequent shift in the sampling events) on growth (weight and length) and to investigate main effects on growth (weight, length, SGR, K) and skeletal anomalies when interaction was not present. All skeletal anomalies data were transformed to achieve homogeneity of variance and normal distribution of residuals by $\arcsin\sqrt{p}$, where p is the proportion of each anomaly type. A linear mixed effect (LME) model was used to investigate the effect of ploidy and temperature and their interaction on total number of vertebrae, total number of deformed vertebrae in deformed individuals, number of deformed individuals and prevalence of compressions and fusions in the second experiment and tank was used as random effect. We considered for all tests performed tank as experimental unit. All data are reported as mean \pm SEM. All data analysis was performed using SPSS version 20.0 (IBM, SPSS Inc.) with a significance level of $P < 0.05$.

2.4 Results

2.4.1 Mortality – incubation

At the end of incubation, mortality was significantly ($P < 0.001$) higher in triploid salmon ($40.9 \pm 0.4\%$) compared to diploids ($31.9 \pm 0.4\%$).

2.4.2 Mortality and growth performance – Experiment 1

At the end of first experiment (2400° days) mortality was not significantly different between ploidies and was $5.8 \pm 3.1\%$ overall. Throughout the experiment, diploids had a significantly ($P < 0.05$) higher mean weight than triploids (Fig. 2.1). Diploids had a significantly ($P < 0.05$) higher mean length than triploids on three sampling events, although there was no significant difference at end of the experiment (final FL diploids 89.4 ± 1.2 mm, triploids 88.2 ± 1.2 mm). However, SGR was not significantly different between ploidies and for both was $1.3 \pm 0.0\%$ day⁻¹.

2.4.3 Mortality and growth performance – Experiment 2

At the end of the second experiment mortality was $2.9 \pm 1.3\%$ overall and significantly ($P < 0.05$) higher in triploid ($3.8 \pm 1.0\%$) than diploid ($1.9 \pm 0.5\%$) salmon. There was no interaction between ploidy and temperature on mortality rate. In the standard treatment, weight and fork length (FL) were not affected by ploidy (Fig. 2.2a) (final size: diploids 49.0 ± 1.2 g and 153.5 ± 1.3 mm; triploids 51.9 ± 1.4 g and 155.3 ± 1.4 mm). In contrast, triploids had significantly ($P < 0.05$) higher mean weight and length than diploids in the elevated treatment on three occasions, the first, second, and last ‘extra’ sampling (Fig. 2.2b) (final size: triploids 62.7 ± 1.5 g and 163.5 ± 1.3 mm; diploids 56.9 ± 1.4 g and 158.1 ± 1.2 mm). In the period between the first and last sampling (included extra sampling in the elevated treatment), no significant interaction between ploidy and temperature was found on weight, length, SGR and K (in the standard treatment both had SGR $2.9 \pm 0.1\%$ day⁻¹ and K 1.3 ± 0.0 and in the elevated treatment

both had $\text{SGR } 3.1 \pm 0.1\% \text{ day}^{-1}$ and $K 1.4 \pm 0.0$). Nevertheless, diploids in the elevated treatment had a significantly ($P < 0.05$) higher mean weight than in the standard treatment and SGR and K were significantly ($P < 0.05$ and < 0.001 , respectively) higher for both ploidies in the elevated treatment than in the standard treatment.

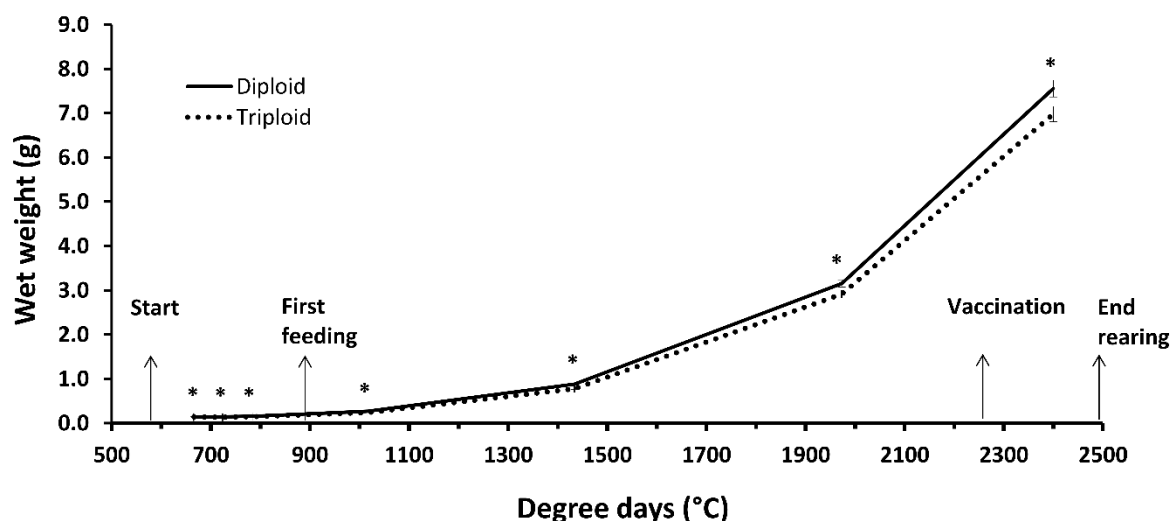


Fig. 2.1. Wet weight (g, mean \pm SEM) in relation to degree days ($^{\circ}\text{C}$ days) during first experiment of triploid and diploid Atlantic salmon *Salmo salar* fry/parr indicating important events (arrows) and statistical differences between ploidies within sample event (asterisks) (one-way ANOVA, $P < 0.05$).

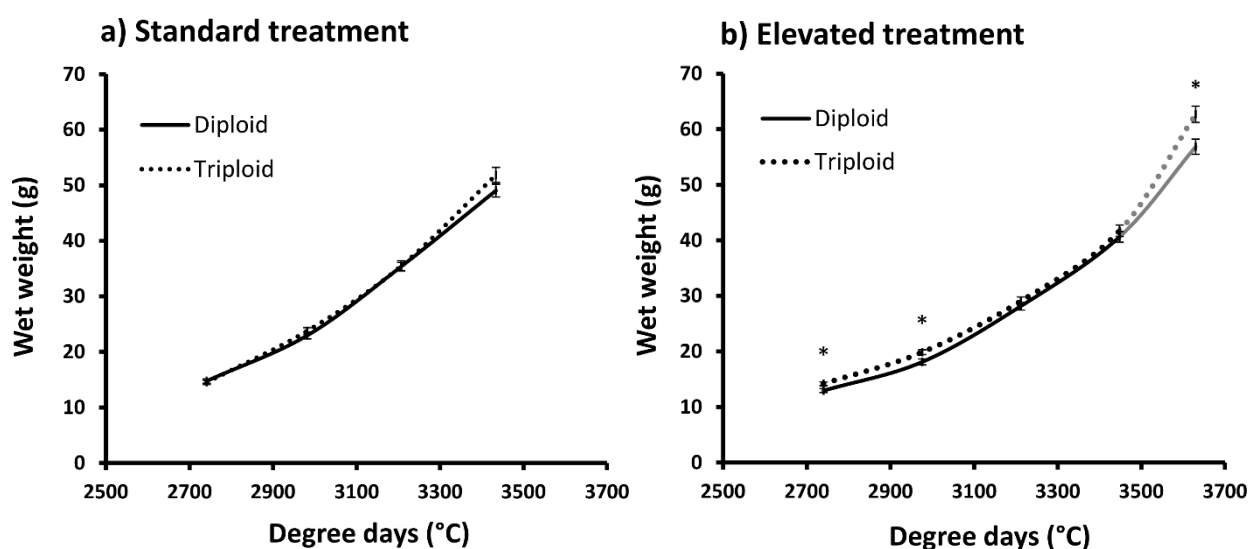


Fig. 2.2. Wet weight (g, mean \pm SEM) in relation to degree days ($^{\circ}\text{C}$ days) during the second experiment of triploid and diploid Atlantic salmon *Salmo salar* parr/pre-smolt for (a) standard (14°C) and (b) elevated (18°C) treatments indicating statistical differences between ploidies (asterisks) (one-way ANOVA, $P < 0.05$).

2.4.4 Prevalence of skeletal anomalies – Experiment 1

Fish visually assessed for skeletal anomalies at 1973° days (~3 g) and 2400° days (~8 g) showed no significant difference between ploidies in the prevalence of all the skeletal anomaly categories. Fish were mostly affected by opercular shortening, in particular by SOB (Fig. 2.3). The other common anomalies were SJ and SOL. At 2400° days the prevalence of SOB in both ploidies decreased compared to that observed at 1973° days and the same was observed for SJ but only in diploids (Fig. 2.3). Conversely, the prevalence of SOL, SOR, and Normal increased (Fig. 2.3). Considering the three opercular shortening categories combined (SOR + SOL + SOB), there was no difference between ploidies during this phase. LJD, Sp DEF and MA were first observed with low prevalence at 2400° days. LJD and Sp DEF were recorded exclusively in triploids at 2400° days, with 1.7% and 0.8% prevalence, respectively.

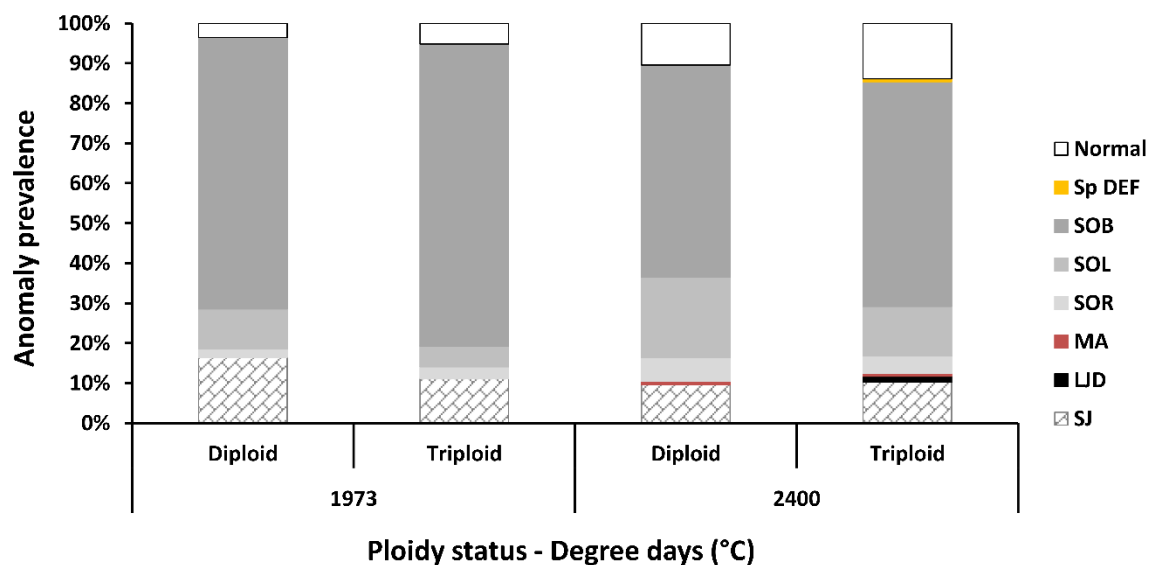


Fig. 2.3. Prevalence of skeletal anomalies (% , mean) in diploid and triploid Atlantic salmon *Salmo salar* fry/parr observed during last two samplings of first experiment indicating the prevalence of each category of the total of individuals visually assessed (abbreviations described in the text, page 38).

2.4.5 Prevalence of skeletal anomalies – Experiment 2

Regardless of ploidy and temperature treatment, throughout the experiment fish were mostly affected by opercular shortening in particular by SOB (Fig. 2.4). Other anomalies recorded

included SOL, SJ, LJD affecting mostly triploid individuals and SOR (Fig. 2.4). In all the individuals assessed the prevalence of fish from both ploidies displaying MA and Sp DEF was low (Fig. 2.4). Over time, the prevalence of SOB in the standard treatment seemed to increase slightly in both ploidies (Fig. 2.4). Conversely, in the elevated treatment SOB remained steady. A decrease over time in the prevalence of SOL was observed in both ploidies for both temperature treatments although not so evident in diploids in the elevated treatment (Fig. 2.4). Prevalence of SOR was steady throughout the experiment in both ploidies in the standard treatment but increased over time in both ploidies in the elevated treatment (Fig. 2.4). MA prevalence differed between ploidies and treatments although the low prevalence did not allow the description of a clear trend (Fig. 2.4). The prevalence of LJD showed the most pronounced trend. In diploids, in both temperature treatments, LJD decreased over time from a maximum of 2.7% to 0.0% on the last sampling event (Fig. 2.4a). On the contrary, in triploids in both temperature treatments LJD prevalence increased to > 10% within the same period (Fig. 2.4b). SJ prevalence decreased over time in both ploidies in the elevated treatment, but decreased during the first three sample occasions in the standard treatment in diploids, then increased in the last sampling event (Fig. 2.4). Finally, increasing prevalence over time of Normal occurred in the elevated treatment for both ploidies (Fig. 2.4).

At the fourth and final sampling event (at approximately the same age ~3400° days) for both ploidies and temperatures, prevalence of SJ and SOR was significantly ($P < 0.05$) higher in diploids at both temperatures with LJD affecting significantly ($P < 0.05$) more triploids likewise at both temperatures (Fig. 2.5). In particular, LJD was the only category on which the effect of the ploidy on the prevalence was strong as highlighted by its absence in diploids. Furthermore, significant differences between ploidies were observed in the elevated treatment in the prevalence of SOL being higher in triploids than diploids and of SOB higher in diploids than triploids (Fig. 2.5). Finally, among triploids the prevalence of SOB was higher in the

standard treatment compared to the elevated treatment, and the prevalence of Normal in the elevated treatment was greater than that in the standard treatment (Fig. 2.5). The prevalence of the three opercular shortening categories combined (SOR + SOL + SOB) was not affected by ploidy, temperature or their interaction. No differences between ploidies and treatments were found for all the other categories (Fig. 2.5).

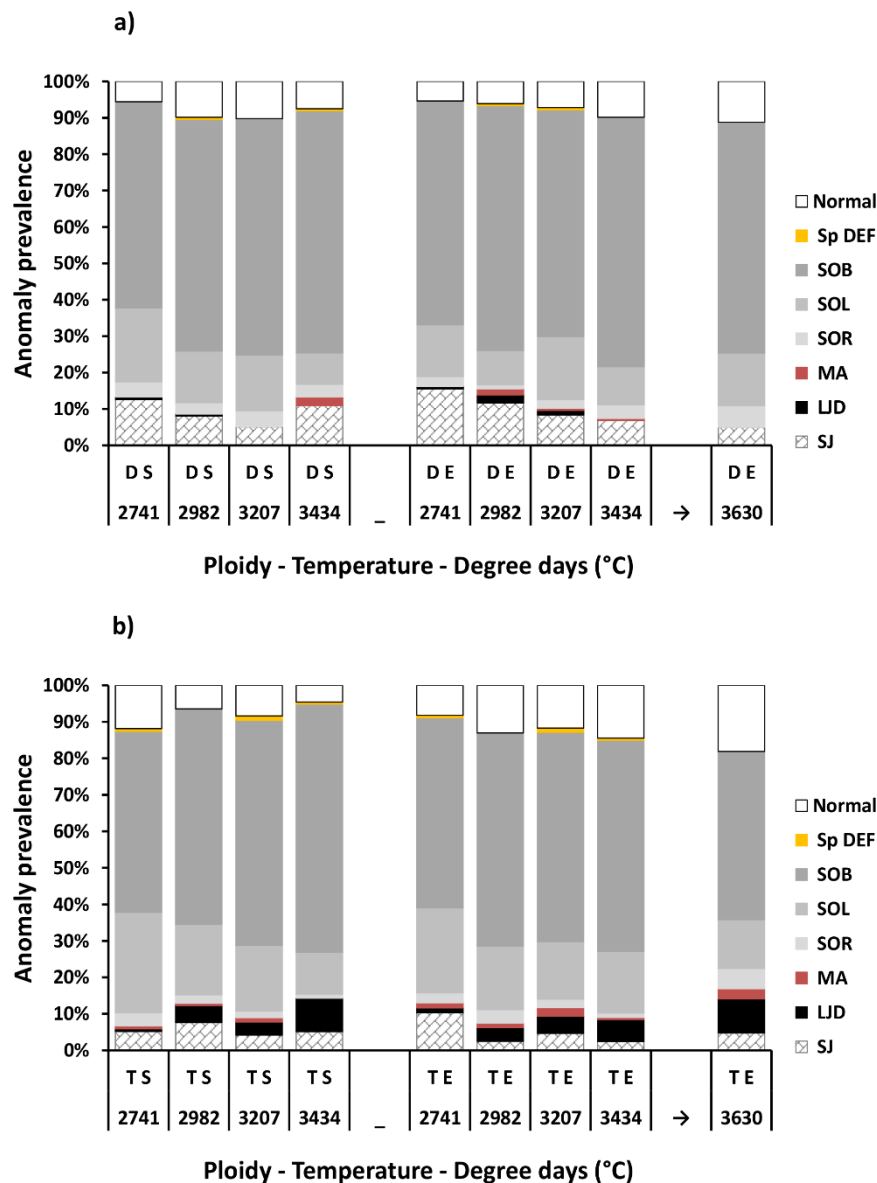


Fig. 2.4. Prevalence of skeletal anomalies (% , mean) in a) diploid (D) and b) triploid (T) Atlantic salmon *Salmo salar* parr/pre-smolt observed throughout the second experiment. Temperatures were Standard (S – 14°C) and Elevated (E – 18°C) and age is indicated in °C days. The fifth sampling for both ploidies in the Elevated treatment is included following the arrow (greater age in °C days, but same number of days in the temperature treatment). Correspondent ages in °C days between temperature treatments have been reported here as equal although slightly different to facilitate comparisons (refer to Table 2.1 for exact values). (Abbreviations described in the text, page 38).

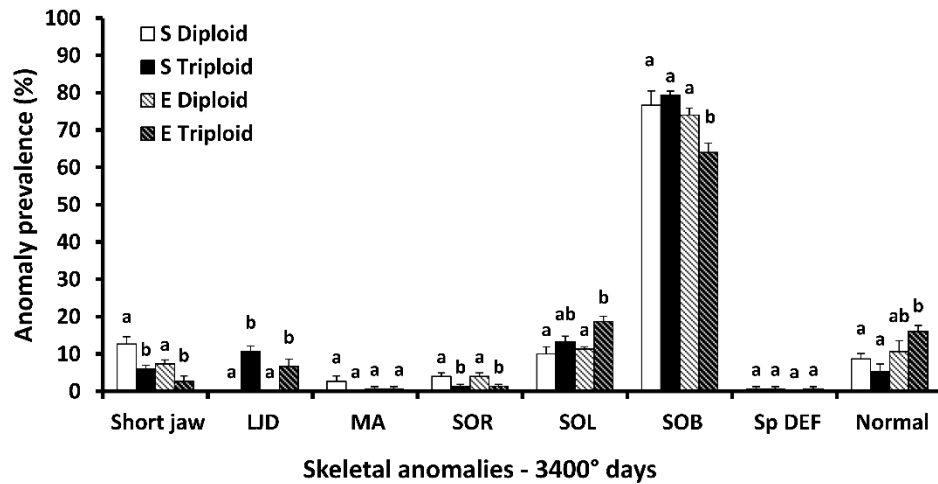


Fig. 2.5. Prevalence of skeletal anomalies (%; mean + SEM) in Atlantic salmon *Salmo salar* parr/pre-smolt observed during the last sampling at the same age (~3400° days) in both treatments (S, standard and E, elevated). For each anomaly category, means were compared between different ploidies in the same temperature treatment and for the same ploidy between the two temperature treatments. Means with different letters are significantly different from one another (two-way ANOVA for interaction and one-way ANOVA for main effects, $P < 0.05$) (Abbreviations described in the text, page 38).

2.4.6 Radiological assessment – Experiment 2

In both temperature treatments, triploids had a significantly ($P < 0.001$) lower number of vertebrae compared with diploids (standard diploids 59.8 ± 0.1 and triploids 59.1 ± 0.1 ; elevated diploids 59.4 ± 0.1 and triploids 58.8 ± 0.1) and temperature had an effect only on diploids with a significantly ($P < 0.05$) lower number of vertebrae in the elevated treatment compared to the standard treatment. The number of deformed triploid individuals (≥ 1 deformed vertebrae) was significantly ($P < 0.05$) higher than diploids in both temperature treatments (standard diploids $3.3 \pm 1.4\%$ and triploids $11.7 \pm 2.7\%$; elevated diploids $6.7 \pm 3.6\%$ and triploids $23.3 \pm 4.9\%$). Despite the higher prevalence in the elevated treatment in both ploidies, compared with the standard treatment, there was no statistically significant effect of temperature or of the interaction between ploidy and temperature on the occurrence of vertebral deformities. No significant difference between ploidy and temperature treatments was found in the number of deformed vertebrae per deformed individual (standard diploids 2.0 ± 0.7 and triploids 2.9 ± 0.4 ; elevated diploids 2.5 ± 0.5 and triploids 2.4 ± 0.3).

The most affected region in diploids in both temperature treatments was R2 while in triploids R4. In the standard treatment, diploids had deformed vertebrae only in R2 and triploids in R2 and R4 with the latter showing the highest prevalence of deformities (Fig. 2.6a). In the elevated treatment, the number of regions affected increased in diploids compared to the standard treatment (Fig. 2.6b). Furthermore, triploids had new vertebrae affected and in a higher prevalence compared to the standard treatment (Fig. 2.6b). Finally, triploids had a significantly ($P < 0.05$) higher prevalence of compressions (standard diploids 1.0 ± 0.0 and triploids 2.0 ± 0.6 ; elevated diploids 2.0 ± 0.0 and triploids 3.7 ± 0.7) and fusions (standard diploids 0.0 ± 0.0 and triploids 1.3 ± 0.3 ; elevated diploids 1.0 ± 0.0 and triploids 2.0 ± 0.8) than diploids in both temperature treatments and no effect of the temperature was observed.

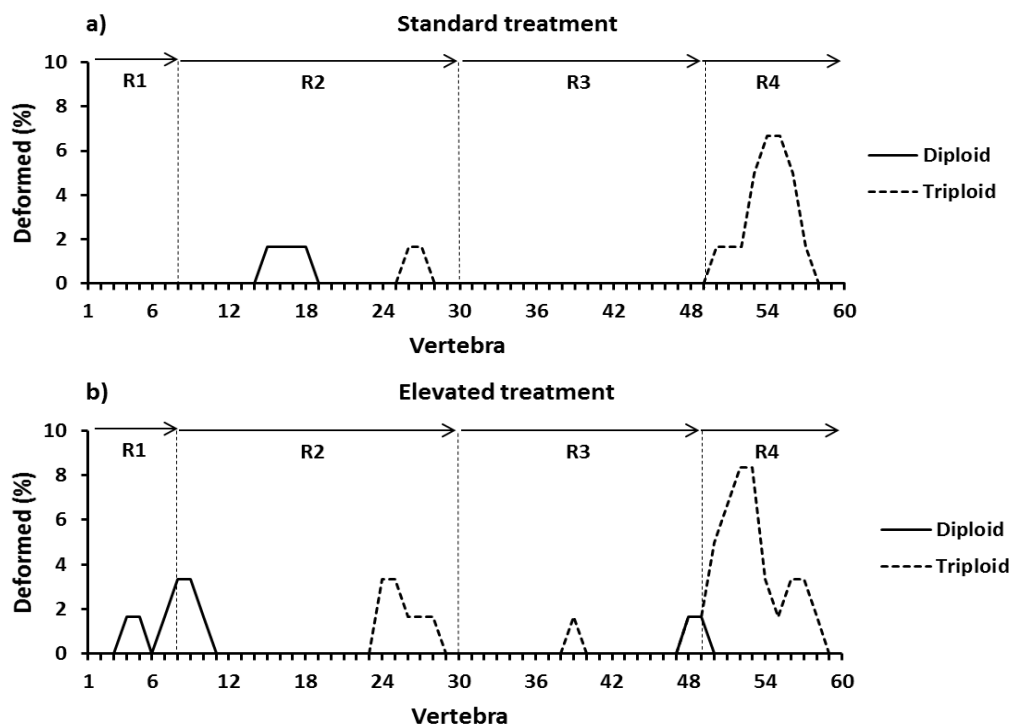


Fig. 2.6. Regions (R) of the vertebral column with deformed vertebrae (%) in diploid (solid line) and triploid (dashed line) Atlantic salmon *Salmo salar* pre-smolt observed at the end of the second experiment after approximately the same days (66 and 63, respectively) spent in (a) standard treatment (14 °C) and (b) elevated treatment (18 °C), $n = 60$ per ploidy per temperature treatment. Regions according to Kacem et al. (1998); R1 = cranial trunk, R2 = caudal trunk, R3 = tail region and R4 = tail fin.

2.5 Discussion

2.5.1 Mortality

In our study, mortality during embryonic and larval stages (from fertilisation to hatching, up to first feeding) was higher in triploid Atlantic salmon compared to diploids and similar during early rearing in accordance with several studies (Sutterlin *et al.*, 1987; McGeachy *et al.*, 1995; O'Flynn *et al.*, 1997; Benfey, 2001). Others studies found no differences between ploidies (Leclercq *et al.*, 2011; Taylor *et al.*, 2011; Taylor *et al.*, 2013) or higher mortality in triploids during both phases (Galbreath *et al.*, 1994; Cotter *et al.*, 2002; Fraser *et al.*, 2014a). Mortality in both ploidies occurred in the period from fertilisation to first feeding and in the following phases it was generally low and decreasing over time in accordance with that reported by most of the cited studies. During the second experiment, it was higher in triploids than diploids at both temperatures, in accordance with mortality in the same stage of development reported by Cotter *et al.* (2002).

2.5.2 Growth

In our assessment during the first experiment, diploids performed better than triploids in terms of initial and final weight. Nevertheless, both ploidies had the same growth rate. Triploids generally have lower weight compared to diploids during the larval stage up to several weeks or months after first feeding when triploids are usually of equal size (Carter *et al.*, 1994; Galbreath *et al.*, 1994; McGeachy *et al.*, 1995; O'Flynn *et al.*, 1997; Cotter *et al.*, 2002; Oppedal *et al.*, 2003; Taylor *et al.*, 2011). A possible explanation for triploids being smaller than diploids after first feeding may lie in differences in distribution in the water column and tank and consequent access to feed (Jungalwalla, 1991; McGeachy *et al.*, 1995) or differences in food consumption compared to diploids (Carter *et al.*, 1994). In the present study, at first feeding diploids started to swim up to the surface and accept feed approximately one week earlier than triploids (unpublished data). In addition, triploids fed only at the bottom of the tank

and frequently rejected feed particles. Similar observations on behavioural differences at first feeding have been reported previously (Jungalwalla, 1991; McGeachy *et al.*, 1995; Cotter *et al.*, 2002).

At the end of the second experiment, Atlantic salmon of both ploidies displayed higher SGR and K in the elevated treatment compared to the standard treatment, in accordance with previous studies showing enhanced growth performance in freshwater at higher temperatures (Solbakken *et al.*, 1994; Forseth *et al.*, 2001; Handeland and Stefansson, 2001; Jonsson *et al.*, 2001; Handeland *et al.*, 2003; Handeland *et al.*, 2008; Grini *et al.*, 2011). In contrast, Atkins and Benfey (2008) found no difference in performance between temperatures testing the same regimes as our study. The current study also showed, in the elevated treatment during the second experiment, that triploids achieved even higher weight and length than diploids at the end of the freshwater phase. Nevertheless, while triploids had no difference in weight between temperature treatments, diploids had higher weight in the elevated treatment compared to standard treatment. This finding implies that although triploids seem to perform better than diploids when exposed to higher temperature, diploids may generally deal with higher temperature better than triploids. Fraser *et al.* (2012a) reported that generally triploids have a lower tolerance to temperature stress than diploids. This lower tolerance may lead to repercussions on growth performance as well as health issues in triploid Atlantic salmon.

2.5.3 Prevalence of skeletal anomalies

Opercular shortening was the most common skeletal anomaly observed in this study. Opercular shortening may result from different degrees of erosion, missing bones, or bones that do not develop normal size or shape of the operculum, and leave the gill filaments uncovered (as reviewed by Boglione *et al.*, 2013b). Opercular shortening has been shown not to affect fish performance in particular SGR (Sadler *et al.*, 2001). Nevertheless, exposure of gills due to lack or damage of opercular plate may affect respiration and marketability of the product (Beraldo

and Canavese, 2011). In the present study, the prevalence was high in comparison to previous studies (as reported below) and the cause(s) was not determined. Ploidy had no effect upon the prevalence of opercular shortening (considering or not the side affected) during first experiment, although diploids and triploids had a different prevalence of unilateral or bilateral opercular shortening in the second experiment. Our study suggests that the side affected by opercular shortening may be influenced over time by both ploidy and temperature. SOR was higher than SOL for both experiments suggesting that environmental conditions (i.e. hydrodynamics of the tanks and side of distribution of feed) may have exposed the right more than the left side of the body of some individuals to the factor causing the shortening of the operculum. Nevertheless, SOB being the most prevalent category from the beginning implies that the conditions mentioned above affected mainly both sides. These findings are difficult to compare with other studies that usually do not report the side affected. Nevertheless, not considering the side, some studies found higher prevalence either in diploids (Taylor *et al.*, 2013) or in triploids (Sadler *et al.*, 2001). Similarly to other authors we found no difference between ploidies (Sutterlin *et al.*, 1987; Taylor *et al.*, 2011; Taylor *et al.*, 2012; Fraser *et al.*, 2013; Fraser *et al.*, 2015) suggesting that localized conditions are important.

Opercular shortening was initially apparent after first feeding (932° days, 1 week after first feeding) in accordance with previous studies (MacLean, 1999; Sadler *et al.*, 2001; Kazlauskienė *et al.*, 2006; Taylor *et al.*, 2011). A similarly high prevalence of opercular shortening was reported by Sutterlin *et al.* (1987) and MacLean (1999) (up to ~90%) and Kazlauskienė *et al.* (2006) (~85%). Nevertheless, MacLean (1999) observed that tagged Atlantic salmon recovered completely from opercular shortening (from ~90% to 0%) as the operculum grows back over time. In other studies, opercular shortening has usually been found at low prevalence and not higher than ~20% (Sadler *et al.*, 2001), although in some cases the data are not clear since opercular shortening was included within the total anomalies and the

actual number (or proportion) of fish affected was not reported (Fjelldal *et al.*, 2007b; Taylor *et al.*, 2011; Taylor *et al.*, 2012; Fraser *et al.*, 2013; Taylor *et al.*, 2013; Fraser *et al.*, 2015).

Opercular shortening is ascribed to environmental factors and culture conditions (as reviewed by Boglione *et al.*, 2013b). The variability in the prevalence of opercular shortening between studies may be explained by different culture conditions applied among experiments (e.g. water, light, flow, stocking density), the quality of the broodstock, or an unbalanced supply of dietary elements (Sutterlin *et al.*, 1987; Sadler *et al.*, 2001; Kazlauskiene *et al.*, 2006). In support of a possible effect of culture conditions, MacLean (1999) found that the occurrence of opercular shortening was due to aggression between fry possibly caused by inadequate distribution of food either in space or time. Furthermore, Kazlauskiene *et al.* (2006) found high prevalence of opercular shortening in hatchery reared Atlantic salmon derived from wild broodstock and not from an industrial selected broodstock. Recently, Taylor *et al.* (2012) suggested that opercular shortening in Atlantic salmon is caused by physical erosion of the operculum due to fish behaviour or water flow. In particular, anecdotal evidence from two commercial hatcheries (N. Ruff and S. Slevic, pers. comm.) suggests the anomaly may be caused by the nipping of the operculum triggered by first feeding, continuing in later stages due to feeding competition and aggressiveness, and exacerbated by bacterial infections. In the present study the use of crates, floating mesh cages and finally tanks over the two experimental phases may have facilitated the occurrence of opercular shortening. In particular, we used a stocking density higher than that recommended for Atlantic salmon (50 kg m^{-3} vs. $20\text{--}30 \text{ kg m}^{-3}$ for fish up to 30 g) (RSPCA, 2015) during the first part of early rearing likely exacerbating the effects of aggressive behaviour manifested at and after first feeding. Furthermore, in the second experiment, the possible effect of temperature, ploidy and their interaction on behavioural differences, with consequent fluctuation in opercular shortening prevalence,

should be taken into account. Further research is needed to ascertain the cause and impact of congenital and environmental triggers for these anomalies.

A SJ was the second most prevalent skeletal anomaly in the study, and in triploids the prevalence decreased over time suggesting that SJ may heal or change to a different anomaly. The concurrent decreasing prevalence of SJ and increasing LJD in triploids implies that for some individuals LJD may be displayed initially as a SJ that over time grows with a downward curving trajectory. The relationship between SJ and LJD remains to be verified through a study that tracks the jaw morphology of individuals of both ploidies with age. In the present study, SJ was observed with different degrees of severity ranging from fish having the upper teeth exposed at the tip of the maxillary to fish with the lower jaw less than half of the length of the upper jaw and with the palate well exposed when the jaw was closed. SJ was specifically reported in only four studies of Atlantic salmon (Sadler *et al.*, 2001; Leclercq *et al.*, 2011; Taylor *et al.*, 2013; Fjellidal *et al.*, 2016) with prevalence, although not individually specified, < 10%. Shortness of lower jaw (dentary bone) has been reported for marine finfish species at hatchery in Europe and Australia (as reviewed by Boglione *et al.*, 2013b; Cobcroft and Battaglione, 2013). In Atlantic salmon the causes of the anomaly are not clear and it is suggested they may be either genetic or related to nutritional deficiency (Sadler *et al.*, 2001). A SJ has been associated with an excess of dietary vitamin A in gilthead sea bream (*Sparus aurata* L.) (Fernández *et al.*, 2008) and European sea bass (*Dicentrarchus labrax* L.) (Mazurais *et al.*, 2009) and with walling behaviour causing physical damage in yellowtail kingfish (*Seriola lalandi* Val.) and striped trumpeter (*Latris lineata* Forster) (Cobcroft *et al.*, 2004; Cobcroft and Battaglione, 2009).

Lower jaw deformity and misaligned jaw (MA) were first noticed from 2400° days (~8 g) in triploids and both ploidies, respectively. The first occurrence of LJD in freshwater was in accordance with some studies (Sutterlin *et al.*, 1987; O'Flynn *et al.*, 1997; Sadler *et al.*, 2001;

Fjelldal *et al.*, 2016). Others observed the anomaly in the seawater phase only, likely because the assessment of anomalies was often first carried out in that phase (Bruno, 1990; Jungalwalla, 1991; Hughes, 1992; King and Lee, 1993; Lee and King, 1994; Quigley, 1995; McGeachy *et al.*, 1996; Leclercq *et al.*, 2011; Fraser *et al.*, 2013; Taylor *et al.*, 2013; Fraser *et al.*, 2015). These findings suggest that the time of LJD onset, in some individuals, may be early during the freshwater phase and given the low prevalence when first displayed, a high number of samples is required for accurate visual detection. During the second experiment, LJD prevalence was similar to the findings of Sadler *et al.* (2001) in all-female triploids (~8%) in a study of Tasmanian populations. Sutterlin *et al.* (1987) suggested that the method used to induce triploidy (heat shock instead of a pressure shock) may impact the prevalence of LJD in triploid salmon parr observed from 5 to 10 cm length (from 26% to 51%). We found a progressive pattern in the increasing prevalence of LJD, also observed by Sadler *et al.* (2001) and Hughes (1992), implying that the anomaly may occur also later in development. In contrast, Fjelldal *et al.* (2016) observed a decrease in the prevalence of LJD after transfer to sea water, and some individuals appeared to shift from LJD to SJ. The higher prevalence of LJD in triploids is in agreement unanimously with previous studies (Jungalwalla, 1991; Hughes, 1992; King and Lee, 1993; Lee and King, 1994; McGeachy *et al.*, 1995; O'Flynn *et al.*, 1997; Benfey, 2001; Sadler *et al.*, 2001; Leclercq *et al.*, 2011; Fraser *et al.*, 2013; Taylor *et al.*, 2013; Fraser *et al.*, 2015; Fjelldal *et al.*, 2016), reinforcing the theory that LJD is mainly related to triploidy. In fact, in the present study and as previously shown, LJD prevalence in diploids was comparatively very low. Lower jaw deformity prevalence/severity is possibly exacerbated by extrinsic factors. These factors may include periods of high growth rate, temperature, diet and congenital predisposition. Fraser *et al.* (2015) found that incubation temperature, and possibly the higher associated growth rate, increased the prevalence of LJD in triploids. Recently, Fjelldal *et al.* (2016) observed a remarkably high occurrence of LJD (up to 90%) in Atlantic

salmon smolts of both ploidies, but again with higher prevalence in triploids than diploids, linked to low dietary phosphorous (P) content (total P, 7.1 g kg⁻¹) from first feeding. In contrast, fish fed a diet with medium or high P content (total P, 9.4 and 16.3 g kg⁻¹, respectively) had an LJD prevalence approaching zero. These findings support the theory that LJD is linked to P deficiency and poor mineralisation in Atlantic salmon as suggested previously by Roberts *et al.* (2001). Nevertheless, in our study although we used commercial diets with declared total P contents of 13 and 17 g kg⁻¹ (values higher than both the medium and the high experimental diets of Fjelldal *et al.*, 2016), the occurrence of LJD was relatively high reaching ~10-11% in triploids of 50-60 g. These findings suggest that although P content in the diet does have an effect on LJD occurrence, the influence of other experimental conditions, such as diet formulation and the resultant P availability and the genetic background of the fish, may lead to different outcomes. In addition, it has been previously shown that elevated temperatures associated with Tasmanian aquaculture of Atlantic salmon impact on nutritional requirements and nutrient utilization (Miller *et al.*, 2006; Carter *et al.*, 2010). Nevertheless, Fjelldal *et al.* (2016) demonstrated that triploid Atlantic salmon have a higher P requirement than diploids, especially during the juvenile stage. Finally, in regards to welfare of fish affected by jaw anomalies, although it has not been investigated whether SJ may impact fish performance, some studies showed that LJD can slow growth down (Bruno, 1990; Burnley *et al.*, 2010) and affect respiration and swimming ability (Lijalad and Powell, 2009). Furthermore, the presence of evident jaw anomalies decreases the value of fish on the market (Benfey, 2001).

Externally visible spinal anomalies were first observed during first experiment at 2400° days (~8 g) and only in a few triploids (< 1%), although several mortalities of both ploidies, especially around first feeding, displayed lordosis, scoliosis, kyphosis (unpublished data). This observation implies that the low prevalence was likely influenced by the mortality of fish with spinal anomalies. In reared sea bass, spinal anomalies have a major impact on survival since

they are often lethal (Koumoundouros *et al.*, 2002). As observed by Sadler *et al.* (2001), the same situation may occur in Atlantic salmon and the greater predisposition of triploids to develop spinal anomalies might contribute to a different mortality rate between ploidies, as observed in the current study. On the other hand, as reported in our study and by Sadler *et al.* (2001) visible spinal anomalies occurring after first feeding tend to be not lethal and are displayed by individuals throughout the development.

The radiological assessment of vertebral deformities revealed that triploid Atlantic salmon had a lower total number of vertebrae than diploids, which is in accordance with recent studies (Leclercq *et al.*, 2011; Taylor *et al.*, 2013; Fraser *et al.*, 2015). Furthermore, we found that an elevated temperature regime applied for just two months in a critical developmental stage (parr/pre-smolt) affected total vertebral number in diploids. This finding suggests that high temperature may affect salmon vertebral body number in a relatively short time in freshwater, not only if applied during incubation as demonstrated by Fraser *et al.* (2015). Nevertheless, since it has been shown that the notochord in Atlantic salmon is segmented, mineralised and vertebral bodies are formed before 2500° days (Grotmol *et al.*, 2003; Grotmol *et al.*, 2005), therefore earlier compared to the age at which different temperature regimes were applied in the present study, it remains unclear the mechanism leading to the loss of one or more vertebral bodies due to the effect of temperature. Conclusive evidence could be achieved only through a longitudinal assessment of vertebral development in individuals raised at different temperatures. A higher prevalence of deformed triploids than diploids (displaying at least one deformed vertebra) is in accordance with all previous studies (Fjelldal and Hansen, 2010; Leclercq *et al.*, 2011; Taylor *et al.*, 2013; Fraser *et al.*, 2014b; Fraser *et al.*, 2015; Fjelldal *et al.*, 2016). Higher prevalence of vertebral deformities in triploids, irrespective of temperature, can be explained by the higher requirement of dietary P that triploids have compared to diploids, particularly during a fast growth period (Fjelldal *et al.*, 2016). Although we found no effect of

temperature and of the interaction between temperature and ploidy on the prevalence of deformed individuals, over time the prevalence of deformed triploids may be exacerbated by high temperature. In fact, high temperature regimes induce vertebral deformities in Atlantic salmon in both freshwater (Ytteborg *et al.*, 2010b) and seawater (Grini *et al.*, 2011). Our finding that there was no difference in the number of deformed vertebrae per deformed individual between ploidies or temperatures is in contradiction with other studies (Ytteborg *et al.*, 2010b; Grini *et al.*, 2011; Taylor *et al.*, 2013; Fraser *et al.*, 2015), but in accordance with Fraser *et al.* (2014b). This can be explained by our assessment being early in the development and that differences in this number are probably displayed later when fish size is larger. In the present study, the low number of deformed vertebrae per deformed individuals implies that at least in this stage welfare of fish may not be affected. Nevertheless, not severe vertebral deformities can worsen and become evident over time (Fjelldal *et al.*, 2007b). Therefore, suitable rearing conditions should be provided from early stages, especially for triploids as more prone to develop vertebral deformities, not to impact fish skeletal development or exacerbate pre-existing conditions leading to both welfare and commercial issues. In fact, in fish displaying severe vertebral deformities growth, swimming performance and metabolism are impaired (Powell *et al.*, 2009; Fjelldal *et al.*, 2012c) and they are usually down-graded at harvest and difficult to be filleted (Sullivan *et al.*, 2007a; Fjelldal *et al.*, 2012c).

The present study showed that triploids had a higher number of regions affected than diploids in the standard treatment but not in the elevated treatment. Furthermore, irrespective of the ploidy, more vertebrae and more regions displayed deformities in the elevated treatment compared to the standard treatment similarly to Fraser *et al.* (2014b). This implies an effect of temperature and as a consequence of higher SGR, as observed in our study, on regional occurrence of vertebral deformities. Other studies found a positive correlation between fast growth and the prevalence of vertebral deformities (Fjelldal *et al.*, 2007a; Hansen *et al.*, 2010;

Grini *et al.*, 2011; Leclercq *et al.*, 2011; Taylor *et al.*, 2013; Fraser *et al.*, 2014b; Fraser *et al.*, 2015). Fjelldal *et al.* (2012c) reported that using high temperature to accelerate growth in Atlantic salmon can affect normal skeletal development. The vertebral tail-fin region (R4) and caudal-trunk region (R2) of both ploidies were the most affected by vertebral deformities in previous studies (Leclercq *et al.*, 2011; Taylor *et al.*, 2013; Fraser *et al.*, 2014b; Fraser *et al.*, 2015). In the present study, the affected regions were distinct for triploids (R4) and diploids (R2). Vertebral deformities, especially in the tail region, can be caused by a dietary P deficiency during the parr stage in triploids (Fjelldal *et al.*, 2016), which implicates dietary phosphorous availability as a potential contributor to the vertebral deformities in the current study. Nevertheless, the time frame of our experiment did not allow detection of possible occurrence of vertebral deformities in R4 in diploids in later stages. The presence of individuals with vertebrae affected in the caudal–trunk region (R2) in freshwater, in correspondence of the dorsal fin, is in accordance with previous studies (Sullivan *et al.*, 2007a; Fjelldal and Hansen, 2010; Grini *et al.*, 2011; Fraser *et al.*, 2014b; Fraser *et al.*, 2015). Similarly to Fraser *et al.* (2014b); Fraser *et al.* (2015), but differently from that reported by Fjelldal *et al.* (2009) and Grini *et al.* (2011), we found fish with vertebral deformities in the caudal–fin region (R3) in freshwater. Fjelldal *et al.* (2009) suggested that deformities in the R3 region likely start in freshwater phase.

2.6 Conclusions

We confirmed that LJD is a predominantly triploid related skeletal anomaly and that there may be a possible developmental link between short jaw and LJD. A deeper understanding of the mechanisms underlying LJD in triploids is needed, potentially investigating changes in gene expression between normal and affected individuals. The unusually high prevalence of opercular shortening occurring in this study, likely triggered by the high stocking density during early rearing, and the effects observed of ploidy, temperature and time on the side

affected, raise the need to identify causal factors in order to prevent this health issue and improve fish welfare. With triploids being most affected by vertebral deformities from the pre-smolt stage, and the effect of elevated temperature on normal skeletal development in both ploidies (number of vertebrae in diploids and regional occurrence), an investigation of optimal temperature regimes, as well as P dietary requirements, for both ploidies during the juvenile (parr/pre-smolt) phase is critical.

2.7 Acknowledgments

This research was supported by the Commonwealth Government's Collaborative Research Network (CRN) Program funding and the Institute for Marine and Antarctic Studies (IMAS). The authors thank Petuna for provision of fertilised eggs and Shaun Slevic and Ryan Wilkinson for their support. Ylenia Pennacchi and Deborah Leonard are thanked for technical assistance during experiments and samplings. Peter de Boer from Mowbray Veterinary Clinic is thanked for providing radiological equipment and assisting with the analysis. GA was supported by a University of Tasmania and IMAS 'Tasmania Graduate Research Scholarship' and JC was supported by a University of the Sunshine Coast CRN Research Fellowship.

CHAPTER 3: Concurrence of lower jaw skeletal anomalies in triploid Atlantic salmon (*Salmo salar* L.) and the effect on growth in freshwater

Gianluca Amoroso^a, Jennifer M. Cobcroft^{a,b}, Mark B. Adams^a, Tomer Ventura^b, Chris G. Carter^a

^a Institute for Marine and Antarctic Studies (IMAS), University of Tasmania

^b Genecology Research Centre, School of Science and Engineering, University of the Sunshine Coast

Keywords: Atlantic salmon, lower jaw, operculum, skeletal anomaly, triploid

This manuscript has been published in *Journal of Fish Diseases* (2016) (online in early view)

DOI: 10.1111/jfd.12492

3.1 Abstract

Triploid Atlantic salmon populations are associated with higher prevalence of lower jaw skeletal anomalies affecting fish performance, welfare and value deleteriously. Anomalous lower jaw can be: curved downward (LJD), shortened (SJ) or misaligned (MA). Two separate groups of triploid Atlantic salmon (~12 g) with either normal lower jaw (NOR) or SJ, were visually assessed four times over three months for presence and concurrence of jaw anomalies (with severity classified) and opercular shortening to understand the relatedness of these anomalous developmental processes. Prevalence of jaw anomalies increased in both groups over time (NOR group - SJ, LJD and MA combined 0 to 24.5%; SJ group - LJD and MA combined 17 to 31%). SJ and LJD occurred both independently and concurrently whereas MA exclusively concurred with them. All three anomalies could be concurrent. Severity of both LJD and SJ increased in the SJ group only. Opercular shortening recovery was observed in both groups but at a slower rate in the SJ group. The SJ group specific growth rate (SGR) was significantly ($P < 0.05$) lower than the NOR group. This study demonstrated the concurrence of SJ, LJD and MA and showed possible deleterious consequences deriving from the condition/s.

3.2 Introduction

The use of triploid Atlantic salmon in aquaculture has been considered for at least 35 years. In fact, triploids present several favourable characteristics for commercial production. The most important are: impaired sexual maturation, consequent prevention of interbreeding with wild populations following farm escapees, similar performance and improved flesh quality (Benfey and Sutterlin, 1984a; Carter *et al.*, 1994; McCarthy *et al.*, 1996; Benfey, 1999; Oppedal *et al.*, 2003; Taylor *et al.*, 2011; Fraser *et al.*, 2012a; Amoroso *et al.*, 2016a; Fjelldal *et al.*, 2016). Nevertheless, a major obstacle to the use of triploids is the higher predisposition to developing skeletal anomalies compared to diploids, these mainly affect the vertebral column and the lower

jaw (Benfey, 2001; Sadler *et al.*, 2001; Leclercq *et al.*, 2011; Fraser *et al.*, 2013; Taylor *et al.*, 2013; Amoroso *et al.*, 2016a; Fjelldal *et al.*, 2016). It is only in Tasmania (Australia) that triploid Atlantic salmon are an important part of commercial production and have been for almost 25 years (Jungalwalla, 1991; Sadler *et al.*, 2000b). In Tasmania, all-female triploid populations have a fundamental role allowing the extension of the harvest period and the supply of a fresh product all year round (Sadler, 2000).

Lower jaw skeletal anomalies in particular, represent a considerable economic loss for producers due to lower survival and growth in affected fish and lower value of the final product because fish can only be filleted and not sold whole (O'Flynn *et al.*, 1997; Benfey, 2001). Furthermore, during the production cycle, fish affected by skeletal anomalies generally undergo intensive and expensive grading (Boglione *et al.*, 2013b; Cobcroft and Battaglione, 2013). Lower jaw skeletal anomalies have been linked to reduced growth and impaired respiration (Bruno, 1990; Lijalad and Powell, 2009; Burnley *et al.*, 2010; Boglione *et al.*, 2013b). A curved downward lower jaw, usually referred to as “lower jaw deformity” (LJD), is the most common lower jaw skeletal anomaly associated with triploid Atlantic salmon and has been reported frequently (Benfey, 2001; Sadler *et al.*, 2001; Leclercq *et al.*, 2011; Taylor *et al.*, 2013; Taylor *et al.*, 2014; Fraser *et al.*, 2015; Amoroso *et al.*, 2016a; Fjelldal *et al.*, 2016). In Tasmania, LJD affects up to 30% of all-female triploid populations (Sadler *et al.*, 2001). LJD consists of different degrees of severity of downward curvature of the lower jaw involving the dentary and glossohyal bones and impairing the functioning of the mouth (Bruno, 1990; Hughes, 1992). LJD appears to be related to different dietary phosphorus (P) requirements in triploids although the underlying mechanisms are not known yet (Fjelldal *et al.*, 2016). In Tasmania, another lower jaw skeletal anomaly commonly found in Atlantic salmon is a shortened lower jaw (SJ) (Sadler *et al.*, 2001; Amoroso *et al.*, 2016a). SJ has been reported mostly in Tasmania and Scotland at different prevalence varying amongst studies, years and

populations and can occur in both diploids and triploids (Sadler *et al.*, 2001; Leclercq *et al.*, 2011; Taylor *et al.*, 2013; Amoroso *et al.*, 2016a). In Tasmania, SJ may affect between 15 and 60% of triploid Atlantic salmon populations (Tasmanian hatchery survey, unpublished data). In fish affected by SJ the lower jaw is not fully developed in length and does not reach the tip of the upper jaw, leaving internal parts of the mouth (i.e. upper teeth and palate) exposed when the mouth is closed, with different degrees of severity (Sadler *et al.*, 2001; Amoroso *et al.*, 2016a). The cause of SJ has not previously been investigated and it is not clear if it might impair fish growth or health. Misaligned jaw (MA) is a lateral displacement of the lower jaw from the longitudinal plane. MA occurs in Tasmanian populations at very low prevalence and is rarely reported in the literature (Bruno, 1990; Sadler *et al.*, 2001; Amoroso *et al.*, 2016a). The cause of MA in Atlantic salmon is not known or whether this condition is concurrent with other anomalies. The same anomaly has been associated with environmental factors in other species (Cobcroft and Battaglene, 2013).

Finally, shortening of one or both opercula commonly occurs in both diploid and triploid Atlantic salmon and consists of different degrees of erosion (but can be also folded or not developed) (Sutterlin *et al.*, 1987; Sadler *et al.*, 2001; Taylor *et al.*, 2012; Amoroso *et al.*, 2016a). The anomaly may expose fish to diseases and impact biological functions, and as a consequence performance, and product saleability (Beraldo and Canavese, 2011; Boglione *et al.*, 2013b; Bruno *et al.*, 2013). Opercular shortening has been anecdotally linked to aggressive feeding behaviour, possibly facilitated by high stock densities, causing physical damage (biting) of the operculum/a (MacLean, 1999; Taylor *et al.*, 2012; Amoroso *et al.*, 2016a) although a definitive cause(s) remains elusive. In Atlantic salmon, recovery from opercular shortening was reported only in one study (MacLean, 1999). Nevertheless, possible factors impeding recovery have never been shown.

Since the underlying reason(s) for lower jaw skeletal anomalies are poorly understood, and given their potential to negatively impact triploid (and diploid) populations, further investigation is required. In particular, in triploids, Amoroso *et al.* (2016a) recently suggested that SJ and LJD may occur concurrently and have a greater impact on the fish. Consequently, the present study tracked the development of all-female triploid Atlantic salmon with normal lower jaw and shortened lower jaw over a three-month period during the juvenile growth phase. The main aim was to verify the concurrence of shortened lower jaw (SJ), curved downward lower jaw or lower jaw deformity (LJD) and misaligned lower jaw (MA) through sequential visual assessments to detect possible developmental patterns indicating dependency or independency of these skeletal anomalies. The severity of the SJ and LJD was also classified in this study and an image analysis technique was tested to confirm visual assessments. Finally, we verified recovery from opercular shortening and assessed the impact of lower jaw skeletal anomalies on the recovery from opercular shortening and growth performance. The findings of the present study provide useful information to improve the knowledge on lower jaw skeletal anomalies in triploid Atlantic salmon whose description and process of occurrence and development require elucidation.

3.3 Materials and methods

3.3.1 Fish source

All-female triploid Atlantic salmon parr (~12 g) were provided by a commercial hatchery in Tasmania on December 2014. All-female embryos were obtained as described by Johnstone *et al.* (1991) and Jungalwalla (1991). Briefly, eggs produced by normal females were fertilised with the milt of sex-reversed genotypic females. Triploidization was achieved 36 min and 15 sec after fertilisation at 8 °C by applying a pressure shock to fertilised eggs for 6 min and 15 sec at 655 Bar (9500 PSI). Fish were incubated at 8 °C and bath-vaccinated for *Yersinia ruckeri* on three occasions (0.25, 1 and 5 g) using Yersiniavac-B (Intervet, Bendigo East, Australia).

From first feeding, fish were exposed to water temperature varying between 12 and 14 °C, a 24 h light photoperiod and fed with Biomar Golden Prima (BIOMAR, Chiloé, Chile). Efficiency of the triploid induction was assessed measuring erythrocyte nuclear length as described by Benfey *et al.* (1984) and confirmed as 100% successful. The selection of fish for the experiment occurred at the hatchery where a batch of fish was randomly collected from the same tank and anaesthetised according to industry practices. Fish were visually assessed and selected depending on the length of the lower jaw, normal or shortened. A lower jaw was considered shortened when its tip was shorter than the tip of the upper jaw with the mouth closed (Fig. 3.1a). At the end of the selection, fish ($n = 192$; 96 per jaw trait) were separately allocated into tubs and transported (3 h) to the aquaculture facilities at the Institute for Marine and Antarctic Studies of the University of Tasmania in Taroona, Tasmania.

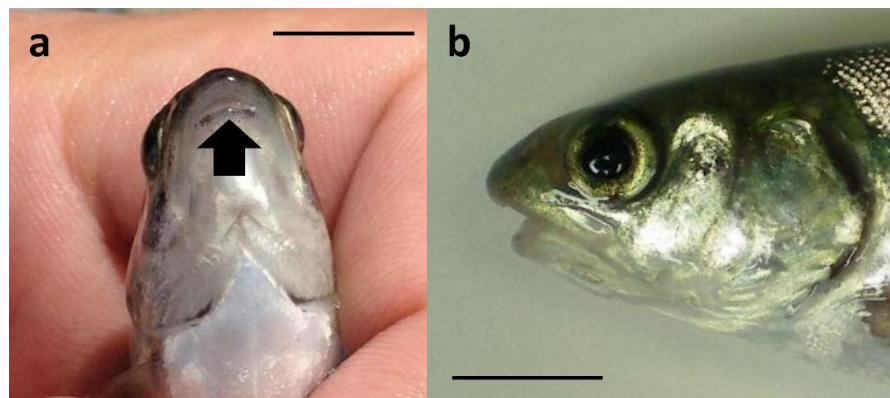


Fig. 3.1. Atlantic salmon (*Salmo salar* L.) parr (approximately 12 g) photographed during fish selection displaying a) shortened lower jaw (SJ) as indicated by the arrow (the upper teeth are exposed and the palate is just visible due to the shortened lower jaw) and b) a concurrence of shortened lower jaw (SJ) and lower jaw deformity (LJD). The individual also presents a slight opercular shortening (scale bars are 1 cm).

3.3.2 Experiment design and conditions

Fish were kept under experimental conditions for three months in a room equipped with twelve 500 L cylindrical tanks in a recirculation system. Four tanks were randomly assigned to each of NOR (normal lower jaw) group and SJ (shortened lower jaw) group and $n = 24$ fish allocated

into each tank according to lower jaw type. Groups were kept separated throughout the experiment and the remaining tanks were run without fish. Stock density was 0.6 kg m^{-3} at the beginning of the experiment and 2.9 kg m^{-3} at the end. Fish displaying a concurrence of a shortened lower jaw (SJ) and lower jaw deformity (LJD) (Fig. 3.1b) were randomly included into the SJ group ($n = 16$ overall) as they were handled. Water exchange (turnover) was maintained at 100% per hour in the tanks and 10% replaced daily in each system with mains water dechlorinated with an appropriate amount of sodium thiosulphate. Temperature was recorded daily and maintained at $15.3 \pm 0.6 \text{ }^{\circ}\text{C}$ throughout the experiment (values for water parameters are reported as mean \pm SD). Dissolved oxygen and pH were recorded daily in the system using Handy Polaris and Handy pH (OxyGuard, Farum, Denmark). Dissolved oxygen saturation was maintained at $98.8 \pm 3.4\%$ and pH 7.1 ± 0.3 . Other parameters were recorded weekly using Freshwater Master Test Kit (API Aquarium Pharmaceuticals, USA) and maintained within safe operating limits: total ammonia – nitrogen (TA-N) $0.9 \pm 1.3 \text{ mg L}^{-1}$, NO_2 $0.2 \pm 0.3 \text{ mg L}^{-1}$ and NO_3 $3.1 \pm 4.1 \text{ mg L}^{-1}$ (Eddy and Williams, 1987). Fish were exposed to a 24-h light photoperiod throughout the experiment using strip LED lights placed on the underside of the tank lids. All fish were fed BIOMAR Golden RC 1.3 (BIOMAR, Chiloé, Chile) in accordance with industry recommendations. Feeds were delivered to satiation at a feeding rate of three times per day for the first month and two times per day for the remaining time. Mortalities were removed and recorded daily and mortality reported as percentage of total dead on total stocked fish. All procedures were carried out with the approval of the University of Tasmania Animal Ethics Committee (approval number A0013044).

3.3.3 Visual assessment of lower jaw skeletal anomalies

All fish were assessed visually at the beginning of the experiment to record the initial prevalence of lower jaw skeletal anomalies. Afterwards, a visual assessment of all fish was performed monthly over three months ($n = 4$ visual assessments overall). The visual

assessments were termed VA1, VA2, VA3 and VA4. At each visual assessment event, except for the last in which fish were euthanized with an overdose of AQUI-S (50 mg L⁻¹), fish were heavily sedated with a dose of 15 mg L⁻¹ of AQUI-S and checked for the prevalence of the following lower jaw skeletal anomaly categories: shortened lower jaw (SJ) (Fig. 3.2), a curved downward lower jaw or lower jaw deformity (LJD) (Fig. 3.2) and misaligned lower jaw (MA) (lower jaw laterally displaced and not aligned on the longitudinal plane). Fish showing a normal lower jaw were classified as normal (Fig. 3.2a). Severity of SJ and LJD was classified within the single category as follows: SJ mild-moderate for fish with the upper teeth exposed and the palate just visible (Fig. 3.1a and 3.2b), SJ severe for fish where the shortened lower jaw clearly exposed the palate (Fig. 3.2c), LJD mild for fish with an angle of downward curvature of the lower jaw (considering the upper jaw as a longitudinal plane and the mouth was in a closed position) between > 0 and $< 45^\circ$ (Fig. 3.2d), LJD moderate between $45-90^\circ$ (Fig. 3.2e) and LJD severe $> 90^\circ$ (Fig. 3.2f). In addition, starting from the second visual assessment (VA2), all fish were checked for prevalence of opercular shortening until the end of the experiment. Each individual fish could be categorised/scored/assessed with more than one anomaly type.

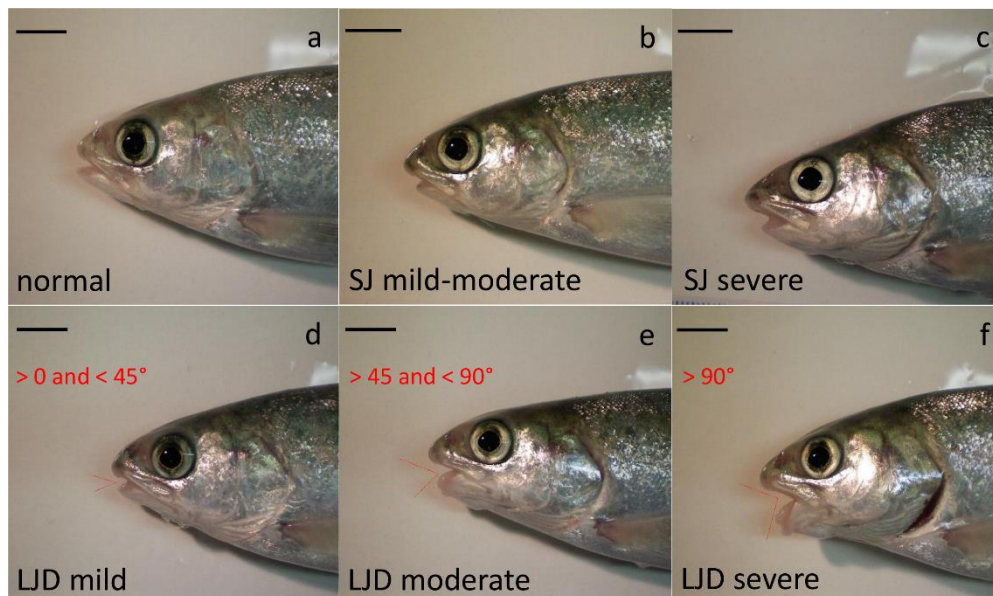


Fig. 3.2. Classification and severity of lower jaw skeletal anomalies affecting Atlantic salmon (*Salmo salar* L.) individuals: a) normal lower jaw, b-c) shortened lower jaw (SJ) with severity discriminated as mild-moderate and severe, d-f) lower jaw deformity (LJD) with three different degrees of severity determined by the angle of downward curvature (values in red text) mild, moderate and severe (scale bars are 1 cm).

3.3.4 Growth and lower jaw index (LJI)

At each visual assessment event, weight (W, g) and fork length (FL, mm) were measured and images taken using a digital photo camera in order to confirm the jaw trait visually assessed and to calculate the ratio of the length of the upper and of the lower jaw by using the lower jaw index (LJI) as described in Lijalad and Powell (2009). Briefly, LJI was calculated for each photographed individual as $LJI = L2 \text{ (pixels)} / L1 \text{ (pixels)}$ where L1 and L2 are the relative length of the upper and the lower jaw (respectively), measured from the insertion point of the pectoral fin to the respective tip. Fish having a $LJI < 0.94$ were considered affected by a lower jaw anomaly (Lijalad and Powell, 2009). The image analysis was performed using Image-J (Schneider *et al.*, 2012). To assess performance, specific growth rate (SGR) and Fulton's condition factor (K) were calculated as follows: $SGR (\% \text{ day}^{-1}) = 100 \times (\ln(W2/W1)) \times d^{-1}$ where W1 and W2 are the initial and final weights (respectively; g) and d is the number of days and $K = W \times 100/FL^3$.

3.3.5 Statistical analysis

The effects of initial jaw trait (two levels, NOR group and SJ group) on mortality and opercular shortening prevalence were tested by generalized linear mixed effect (GLME) models with a binomial distribution, while on growth parameters (W, FL, SGR, K) and LJI were tested by One-way ANOVAs and tank was considered as an experimental unit. All data are reported as mean \pm SEM. All data analyses were performed using SPSS version 22.0 (IBM, SPSS Inc., Chicago, IL, USA) with a significance level of $P < 0.05$.

3.4 Results

3.4.1 Fish performance

Mortality was 3% of the fish initially stocked and not significantly different between the NOR and SJ groups. At VA1 the SJ group had significantly ($P < 0.001$) higher mean weight and FL

than the NOR group (Fig. 3.3a, b). By VA2 there was no difference in FL (Fig. 3.3b), and by VA3 there was no difference in W (Fig. 3.3a). SGR was significantly ($P < 0.05$) lower in the SJ group than in NOR (NOR $2.04 \pm 0.01\%$ day⁻¹; SJ $1.94 \pm 0.02\%$ day⁻¹) and K was not significantly different between groups at the end of the experiment being 1.19 ± 0.01 (average of groups).

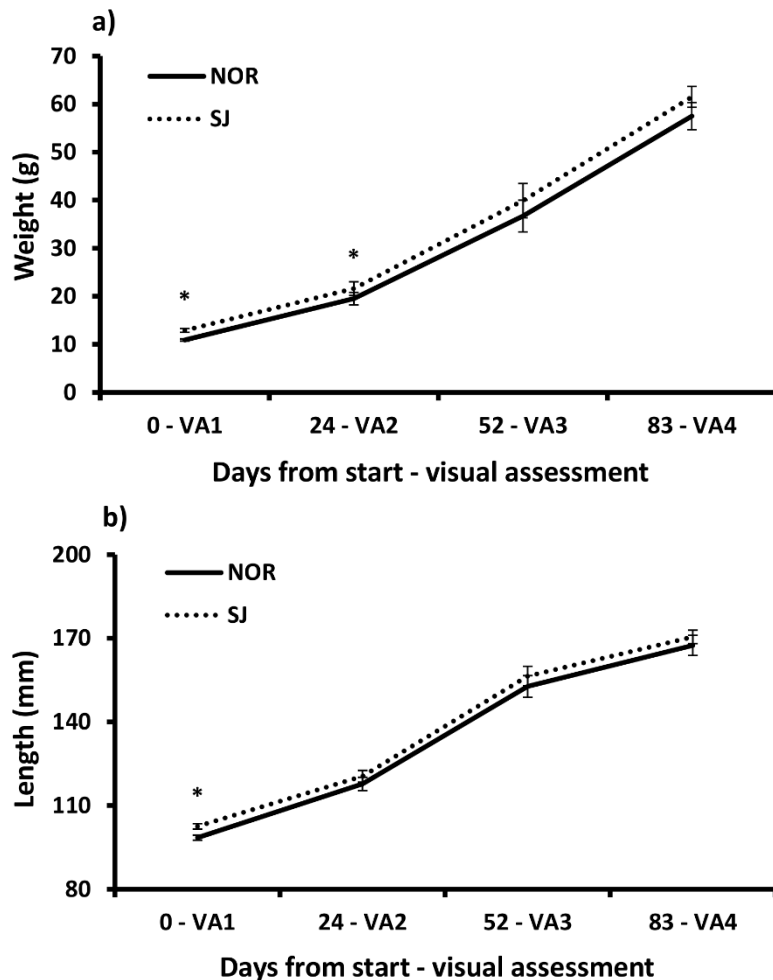


Fig. 3.3. Weight (g) a) and fork length (mm) b) (mean \pm SEM) of Atlantic salmon (*Salmo salar* L.) individuals in the two groups ($n = 96$ per group) NOR and SJ (established at the beginning of the experiment according to the jaw trait initially displayed - normal or shortened lower jaw) during the four visual assessments performed (V1-V4) and corresponding days from the start of the experiment. Significant differences between groups are indicated by asterisks (one-way ANOVA $P < 0.05$).

3.4.2 Prevalence of lower jaw skeletal anomalies

In the NOR group, at VA1 the prevalence of lower jaw anomalies was 0%. The first anomalies were present at VA2 and were SJ mild-moderate and LJD mild (18% and 1% prevalence respectively) and both increased at VA4 (25% and 3% respectively). MA was apparent at VA3 (2%) to remain steady at VA4 (Fig. 3.4). Consequently, the prevalence of normal jaw decreased at VA2 and VA3 (81% to 73%) and increased at VA4 (75%) (Fig. 3.4).

In the SJ group, at VA1 the prevalence of SJ mild-moderate was 100% and the prevalence of LJD mild was 17%. SJ mild-moderate decreased over VA3 and VA4 (90% to 87%). SJ severe was first observed at VA3 (4%) and increased at VA4 (7%) (Fig. 3.4). LJD mild increased at VA2 (23%) and then decreased at VA4 (12%), concurrent with an increase in LJD moderate increasing over VA2 and VA4 (2% to 9%) (Fig. 3.4). LJD severe was observed only at VA4 (2%) (Fig. 3.4). MA was apparent from VA3 (5%) and increased at VA4 (8%) (Fig. 3.4). Fish with a normal jaw were first observed from VA3 (6%) and prevalence remained steady until VA4 (Fig. 3.4).

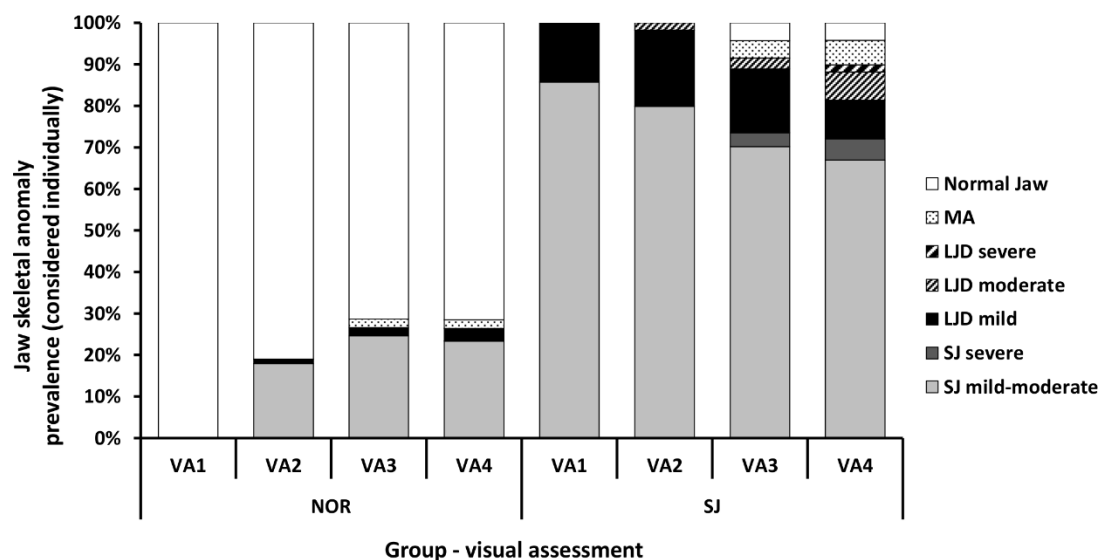


Fig. 3.4. Mean prevalence of lower jaw anomaly categories (considered individually) observed in Atlantic salmon (*Salmo salar* L.) individuals within the two groups ($n = 96$ per group) NOR and SJ (established at the beginning of the experiment according to the jaw trait initially displayed - normal or shortened lower jaw) during the four visual assessments performed (VA1-4). All fish in the SJ group initially had SJ mild-moderate, including those with concurrent LJD mild indicated (black). The dominant single anomaly was used for the classification, where anomalies concurred, with LJD shown in preference to MA and then SJ. For further information refer to concurrence (Fig. 3.5) and types (Fig. 3.6).

3.4.3 Concurrence of lower jaw skeletal anomalies and types

There was no concurrence of jaw anomalies in the NOR group fish up to VA2 (Fig. 3.5). Concurrence was only apparent from VA3 (2%) (Fig. 3.5) with SJ mild-moderate+MA accounted for 100% among the concurring categories (Fig. 3.6). Increase in concurrence was

observed at VA4 (5%) (Fig. 3.5), with SJ mild-moderate+LJD mild accounting for 60% and SJ mild-moderate+MA for the remainder (Fig. 3.6).

In the SJ group, at VA1 the proportion of anomaly displayed individually (83%) and concurrently (17%) (Fig. 3.5) reflected the initial allocation (Fig. 3.4) with SJ mild-moderate+LJD mild accounting for 100% (Fig. 3.6). Concurrence increased during the experiment (from 17 to 29%) (Fig. 3.5) due to the increasing presence of concurring SJ and LJD with different severity (Fig. 3.7 example of SJ severe+LJD mild) as well as MA (Fig. 3.6). The increase in severity for both SJ and LJD (shown previously in Fig. 3.4) was also highlighted by variations in presence over time of more severe concurring categories (Fig. 3.6). In particular, from VA1 to VA4, SJ mild-moderate+LJD mild decreased (100 to 42%) while other categories increased: SJ mild-moderate+LJD moderate (9 to 19%), SJ mild-moderate+LJD severe (0 to 8%), SJ mild-moderate+MA (0 to 19%) and all the remaining (0 to 4%) (Fig. 3.6). MA was the last anomaly to appear over time and contributed to the presence of fish displaying up to three lower jaw skeletal anomalies concurrently (Fig. 3.6). MA was present irrespective of the presence of LJD resulting in fish displaying either SJ+MA or SJ+LJD+MA (Fig. 3.6).

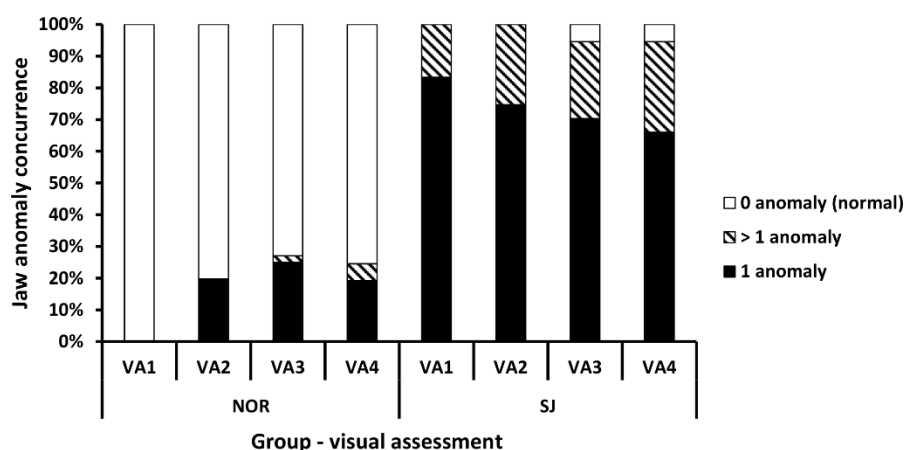


Fig. 3.5. Mean prevalence of Atlantic salmon (*Salmo salar* L.) individuals ($n = 96$ per group) displaying no anomalies (or normal) (white) and one (black) or more lower jaw skeletal anomalies concurrently (black and white gradient), within the two groups NOR and SJ (established at the beginning of the experiment according to the jaw trait initially displayed - normal or shortened lower jaw) during the four visual assessments performed (VA1-4).

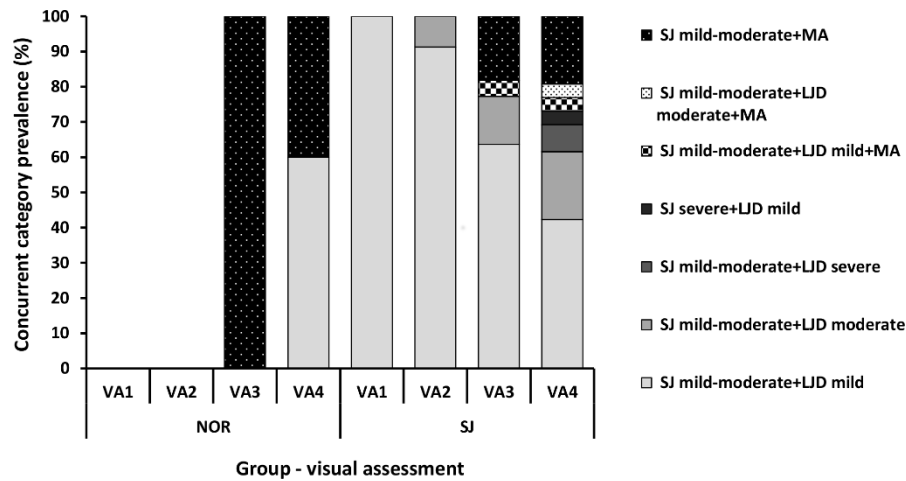


Fig. 3.6. Mean prevalence of lower jaw skeletal anomaly categories displayed concurrently in Atlantic salmon (*Salmo salar* L.) individuals, within the fish that had two or more concurrent anomalies, in the two groups NOR and SJ (established at the beginning of the experiment according to the jaw trait initially displayed - normal or shortened lower jaw) during the four visual assessments performed (VA1-4).



Fig. 3.7. Atlantic salmon (*Salmo salar* L.) (approximately 60 g) displaying a concurrence of severe shortened lower jaw (SJ) and mild lower jaw deformity (LJD) at the end of the experiment (scale bar 1 cm).

3.4.4 Lower jaw index (LJI)

The LJI showed a clear distinction between the NOR and SJ groups. Throughout all four visual assessments the SJ group had a significantly ($P < 0.05$) lower LJI (0.92 ± 0.01) than the NOR group (0.96 ± 0.01). Furthermore, within the NOR group, fish had a significantly ($P < 0.05$) lower LJI at VA4 (0.96 ± 0.01) compared to VA1 (0.97 ± 0.01) and no significant difference in LJI was found for SJ between VA1 (0.92 ± 0.01) and VA4 (0.92 ± 0.01).

3.4.5 Opercular shortening

At VA2 the prevalence of opercular shortening was $31.8 \pm 9.3\%$ and $34.1 \pm 6.3\%$ for NOR and SJ groups, respectively (Fig. 3.8), and was not significantly different between groups. In most

of the fish only a small portion of the operculum, mainly the dorsal region, was affected and the gill filaments were only just visible underneath (Fig. 3.1b). Over time, the prevalence of opercular shortening decreased in both groups, although in the SJ group the decrease was at a slower rate and the NOR group had a significantly ($P < 0.05$) lower prevalence of opercular shortening than the SJ group at VA3 and VA4. At VA4, the prevalence of opercular shortening was $3.1 \pm 1.7\%$ and $13.1 \pm 2.9\%$ for NOR and SJ, respectively (Fig. 3.8). At VA3, where the biggest decrease in prevalence occurred, transparent tissue was observed at the edge of the opercular plate in some fish affected by severe opercular shortening, although the opercular chamber underneath remained visible (Fig. 3.9).

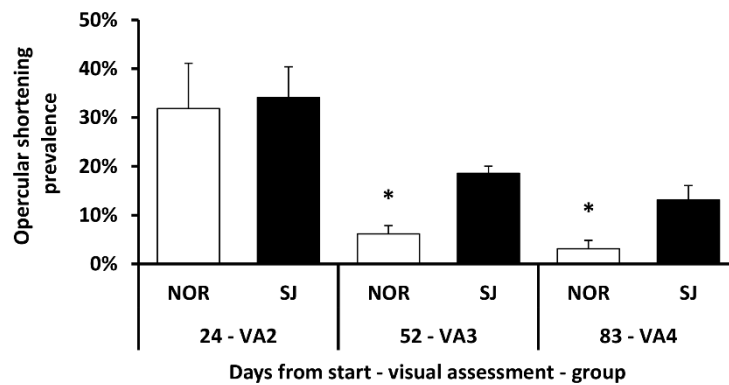


Fig. 3.8. Prevalence (% mean + SEM) of opercular shortening in Atlantic salmon (*Salmo salar* L.) individuals in the two groups normal (NOR) and shortened lower jaw (SJ) (groups established at the beginning of the experiment according to the jaw trait initially displayed - normal or shortened lower jaw) from the first record at sampling event VA2 to the end of the experiment VA4 and corresponding days from start. Significant differences in the prevalence between groups, within the same visual assessment event, are indicated by asterisks (GLME $P < 0.05$).

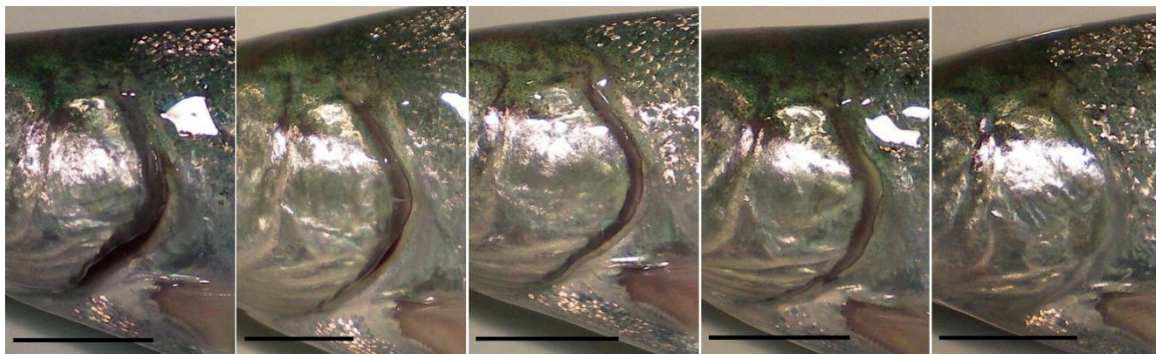


Fig. 3.9. The operculum of Atlantic salmon (*Salmo salar* L.) individuals at same stage of development showing different degrees of recovery from opercular shortening. Transparent tissue was visible at the posterior edge of the affected area, with the opercular chamber underneath still visible. The last individual on the right displays a normal operculum (scale bars are 1 cm).

3.5 Discussion

The present study has shown that in triploid Atlantic salmon SJ and LJD concur and that the lower jaw may undergo dramatic structural changes in a relatively short time during the parr/pre-smolt phase. The possible onset of LJD in fish already displaying SJ was recently suggested by Amoroso *et al.* (2016a). SJ and LJD occurred also independently in both groups demonstrating that both normal and SJ-affected fish can develop LJD. It is not clear whether individuals with LJD may develop a SJ. In the present experiment, the trends in categories prevalence over time (notably increasing LJD and decreasing SJ) suggest that this did not happen. Nevertheless, only an experiment tracking fish individually would enable this to be confirmed. However, it would be difficult to detect the onset of a SJ in fish which already have LJD. In fact, the downward curvature of the lower jaw does not allow alignment of the tip of the lower jaw with the tip of the upper jaw of the fish and as a consequence to detect a shortness just mild. More sophisticated image analysis measurements may be employed to help the detection. Furthermore, research is needed to verify whether the onset of one lower jaw skeletal anomaly may facilitate the development of the other as well as the possible causes leading to the concurrence.

SJ was either first apparent together with LJD or displayed separately (as shown in the NOR group) or LJD occurred later compared with SJ (as shown in the SJ group). Similarly, Amoroso *et al.* (2016a) found that the onset of SJ is usually earlier in development compared to LJD (~3 g vs ~8 g respectively). On the other hand, Sadler *et al.* (2001) reported the opposite with LJD developing earlier than SJ. We found that SJ could also occur later in development (at ~20 g in the NOR group). Similarly, Sadler *et al.* (2001) found that the prevalence of fish with a SJ increased after the transfer to seawater implying occurrence at any time in development (i.e. freshwater and seawater phase). The same may also apply to LJD, although previous studies showed the first evident occurrence to be generally related to a particular

period between first feeding and 8-9 g size (Sadler *et al.*, 2001; Amoroso *et al.*, 2016a; Fjelldal *et al.*, 2016). Furthermore, in the NOR group, LJD was displayed concurrently with SJ only later in development suggesting that in fish with a normal jaw that develops into a SJ further onset of a LJD may be delayed compared to the direct development of LJD from a normal jaw. Amoroso *et al.* (2016a) observed that the prevalence of fish displaying SJ is usually higher compared to LJD at least in freshwater during early rearing and the prevalence remained relatively steady later in development. Conversely, Sadler *et al.* (2001), found in freshwater a lower prevalence of SJ compared to LJD in Tasmanian Atlantic salmon. These findings suggest that prevalence of SJ may vary between years, populations and experimental conditions.

While the occurrence of SJ has been reported in both diploids and triploids at different prevalence (Sadler *et al.*, 2001; Leclercq *et al.*, 2011; Taylor *et al.*, 2013; Amoroso *et al.*, 2016a), LJD has been linked mainly to triploids (Benfey, 2001; Sadler *et al.*, 2001; Leclercq *et al.*, 2011; Taylor *et al.*, 2013; Fraser *et al.*, 2015; Amoroso *et al.*, 2016a; Fjelldal *et al.*, 2016). The difference in occurrence between ploidies suggests that SJ and LJD may be driven by independent developmental pathways with LJD likely driven by predisposition in triploids. For both SJ and LJD the causes are not known. However, LJD has been linked to a lack of dietary phosphorus (Roberts *et al.*, 2001; Fjelldal *et al.*, 2016). The findings of Fjelldal *et al.* (2016) suggest that LJD may occur any time in early development when fish are not supplied with an appropriate amount of dietary P. Furthermore, it has been shown that triploids have a higher dietary P requirement than diploids (Fjelldal *et al.*, 2016; Smedley *et al.*, 2016). Overall in Atlantic salmon, the development of lower jaw skeletal anomalies has been associated with several factors such as genetic predisposition especially in triploids (Sadler *et al.*, 2001), a deficiency in dietary elements (Sadler *et al.*, 2001), high growth rates (King and Lee, 1993; Taylor *et al.*, 2014; Fraser *et al.*, 2015), high incubation temperatures (Fraser *et al.*, 2015) and the different requirements for phosphorus in triploids (Fjelldal *et al.*, 2016). Similarly, in

poultry skeletal defects represent a considerable issue and are generally considered the result of a combination of factors (i.e. genetic, nutrition and management) (Waldenstedt, 2006). Lee and King (1994) suggested that the occurrence of jaw anomalies in Atlantic salmon may be diminished by slowing down the growth rate implying that a fast growth may affect the isometric development of the upper and lower jaw. The high SGR in the present study ($\sim 2\%$ day⁻¹) compared to other recent studies on triploids (Fraser *et al.*, 2013; Taylor *et al.*, 2013) (~ 0.5 and $\sim 1.2\%$ day⁻¹ respectively) could have driven the dramatic structural changes in the lower jaw as well as the occurrence and concurrence of jaw anomalies in a relatively short time even in fish starting with a normal lower jaw. A parallel can be drawn with poultry where it has been shown that high growth rate leads to the occurrence of bone defects and enhances skeletal muscles development to the detriment of bone (Julian, 1998; Rath *et al.*, 2000; Waldenstedt, 2006). The role of a possible imbalance in the mechanical forces exerted by skeletal muscles on bone in the development of lower jaw skeletal anomalies in Atlantic salmon requires elucidation. These findings should be of interest especially for Tasmania where triploid Atlantic salmon may experience faster growth rates due to higher freshwater temperatures compared to the northern hemisphere producers (Battaglione *et al.*, 2008; Carter *et al.*, 2010).

The present study showed that fish can recover from SJ. Nevertheless, further investigation on individually tagged fish would help to understand whether or not fish can recover from all types of severity. Recovery from SJ strongly suggests the presence of mechanisms that restore the balance in growth between the upper and lower jaw. Since severe SJ appeared in later stages within one month between sampling events, it suggests that SJ may be due to an impairment in the growth of the lower jaw tissues and that they may either stop growing/lengthening or grow at a slower/delayed rate compared to the upper jaw. Although recovery from LJD was not observed in the current experiment, recently Fjelldal *et al.* (2016)

observed fish recovering from LJD when fed a diet with medium or high P content (9.4 and 16.3 g kg⁻¹, respectively). This finding suggests that dietary P deficiency may affect recovery from at least one category of lower jaw skeletal anomaly. We suggest that the process of increasing severity of LJD may also be subjected to the same mechanisms of growth impairment as for SJ. In fact, in the present study, LJD severity (from mild to severe) increased, but not in all the fish affected, suggesting that LJD may or may not continue to worsen. Consequently, LJD could remain in a similar condition of severity from when it first occurred to later stages and likely until the adult stage. Nevertheless, in a case of concurrence, a worsening of SJ did not necessarily correspond to a worsening in LJD in the present study, reinforcing the theory that the two anomalies may have different underlying mechanisms and developmental patterns. Further research is needed to understand possible exacerbating factors for the severity of both lower jaw skeletal anomalies.

Malaligned lower jaw (MA) occurred with increasing prevalence exclusively in fish with a lower jaw already affected by an anomaly and mainly by SJ. These findings suggest that the occurrence of a lateral displacement on the longitudinal plane of the lower jaw may be facilitated in a lower jaw having a skeletal structure already compromised (i.e. in both SJ and LJD). It remains unclear whether MA may be the result of physical injury due to weakened structure of the jaw. MA (also known as cross-bite or twisted jaw) has been previously associated in several farmed fish species with environmental (e.g. injury due to walling behaviour) and nutritional factors (e.g. vitamin deficiency) (Boglione *et al.*, 2013b; Cobcroft and Battaglione, 2013). MA has only been reported in three studies of Atlantic salmon to date (Bruno, 1990; Sadler *et al.*, 2001; Amoroso *et al.*, 2016a). Hughes (1992) reported, after histological examination, that the two sides of the lower jaw (specifically the Meckel's cartilage) had a different diameter in triploid Atlantic salmon when compared with diploids. This finding implies both a congenital morphological imbalance of jaw skeletal tissues

underlying the tendency of triploids to developing higher prevalence of lower jaw skeletal anomalies and that in some individuals a lateral displacement of the lower jaw may occur due to that imbalance.

The current experiment demonstrated that the majority of fish from both groups recovered from opercular shortening. Similarly, MacLean (1999) observed complete recovery from opercular shortening in tagged diploid Atlantic salmon over a period of nine months. Opercular shortening is a commonly observed anomaly in Atlantic salmon and recently Taylor *et al.* (2012) suggested that it is not a real skeletal anomaly. Its occurrence has been explained by environmental conditions influencing first feeding triggering abnormal behaviour and aggressiveness and leading to damage of the opercular tissues by fish biting each other (Sutterlin *et al.*, 1987; MacLean, 1999; Sadler *et al.*, 2001; Kazlauskienė *et al.*, 2006; Taylor *et al.*, 2012; Amoroso *et al.*, 2016a). In the present study, the observation of a transparent tissue (possibly the epithelial extremity of the operculum that functions to seal of the opercular chamber during respiration) developing from the affected opercula in some fish suggests that the recovery is through a re-growth of the opercular tissues. A re-growth of opercula has been described in farmed gilthead sea bream (*Sparus aurata* L.) by Beraldo and Canavese (2011) implying the possible presence of a similar process in other fish species. Furthermore, the observation of this tissue only in fish affected by a more severe shortness suggests that, in most of the fish initially affected by a slight shortness, the tissue affected may have grown back relatively quickly (< one month) without allowing its detection between two visual assessment events. Taking into account that opercular shortening may be linked to feeding behaviour and aggressiveness, the very low stocking density (< 3 kg m⁻³) combined with an easy access to feed for fish in the present study, may have facilitated the recovery. Nevertheless, histological characterisation of the tissue is needed to understand the mechanisms involved. In addition, a

possible anatomical threshold or stocking density/feeding rate at which re-growth may not occur should be investigated.

Although not verified through individual tagging, our results suggest that fish affected by a jaw anomaly may have a slower recovery from opercular shortening. In fact, an impairment in the functioning of a fundamental part of the body employed to both feeding and breathing may slow down the process. Nevertheless, individuals affected by jaw anomalies could have also recovered at the same rate but have been more prone to re-develop opercular shortening. In addition, slower recovery may be also due to an intrinsic issue with bone remodelling in the opercular tissues. Sutterlin *et al.* (1987) and Lijalad and Powell (2009) suggested that in Atlantic salmon the presence of a LJD may affect the water flow passing through the gills and as a consequence respiration and fitness. Boglione *et al.* (2013b) reported that fish affected by severe jaw anomalies are less efficient in feeding and have slower growth compared to normal fish. In particular, Bruno (1990) and Burnley *et al.* (2010) observed that Atlantic salmon affected by jaw anomalies had a slower growth compared to fish with a normal lower jaw although the assessment was anecdotal. Recently, Taylor *et al.* (2014) urged the assessment of both growth performance of individuals affected by jaw skeletal anomalies and the effect of growth on the occurrence of jaw skeletal anomalies. In the current experiment, we also suggest that the presence of jaw anomalies may affect growth, in particular SGR, as fish with a normal lower jaw, although starting with a lower mean weight, equalised mean weight and length over time of fish affected by jaw anomalies. These findings imply a likely effect of jaw anomalies on performance although the relationship remains to be validated through an experiment with tagged individuals.

Finally, we showed that LJI can also be successfully employed to detect fish having a SJ, from a relatively early stage (12 g) and possibly from earlier stages. Fish affected by either SJ or LJD had a $LJI < 0.94$ while fish with a normal lower jaw > 0.94 in the current experiment.

In fact, in fish with a LJD an increase in the severity of the downward curvature of the lower jaw corresponds to a retrogression of the position of the tip, resulting in a lower LJI in affected fish. It was difficult to set a threshold to distinguish a SJ mild-moderate from a severe as well as to discriminate between a SJ and a LJD having the same LJI, which can be done only visually with a 3-dimensional perspective of the jaw elements. In the present study, LJI enabled the detection and progression over time of jaw anomalies in the NOR group. In fact, at the end of the experiment the group had a lower LJI compared to the beginning of the experiment. The same did not occur for the SJ group although prevalence and severity of jaw anomalies increased. This finding could be explained by both the appearance over time of fish displaying a normal jaw (i.e. recovering from SJ) likely balancing the initial and final LJI and the fact that, as mentioned above, the backward movement of the tip of the lower jaw occurring during a LJD did not substantially affect the LJI of a fish already displaying a SJ.

3.6 Conclusions

In conclusion we showed that shortened lower jaw (SJ) and lower jaw deformity (LJD) can manifest concurrently and misaligned jaw (MA) is more likely to develop in individuals with jaw structure already compromised. The developmental patterns observed as well as the process of worsening of both SJ and LJD imply an independence of the two lower jaw skeletal anomalies. Further research is needed to understand the factors causing and the mechanisms underlying SJ, LJD and MA in order to prevent them. The changes in prevalence of the different categories, the concurrence of some anomaly types and even recovery from SJ over a relatively short time highlight the plasticity of the lower jaw during this developmental stage and suggest the relationship between fast growth and the occurrence of jaw anomalies should be pursued. We observed that Atlantic salmon can recover from opercular shortening and that jaw anomalies may impede recovery from this condition as well as impact upon general growth performance, although further research is needed to validate these findings. Finally, we found

that image analysis can assist in detecting lower jaw anomalies from early stages and changes in occurrence over time.

3.7 Acknowledgements

This research was supported by the Commonwealth Government's Collaborative Research Network (CRN) Program funding and the Institute for Marine and Antarctic Studies (IMAS). The technical team of the Fisheries and Aquaculture Centre of the Institute for Marine and Antarctic Studies (Taroona) is thanked for their assistance during the experiment and samplings. GA was supported by a University of Tasmania and IMAS "Tasmania Graduate Research Scholarship" and JC was supported by a University of the Sunshine Coast CRN Research Fellowship.

CHAPTER 4: Multigenic delineation of lower jaw deformity in triploid Atlantic salmon (*Salmo salar* L.)

Gianluca Amoroso^a, Tomer Ventura^b, Jennifer M. Cobcroft^{a,b}, Mark B. Adams^a, Abigail Elizur^b, Chris G. Carter^a,

^a Institute for Marine and Antarctic Studies (IMAS), University of Tasmania

^b Genecology Research Centre, School of Science and Engineering, University of the Sunshine Coast

Keywords: Atlantic salmon, lower jaw deformity, triploid, *col2a1*, *gphb5*.

This manuscript has been submitted to PLOS ONE (2016)

4.1 Abstract

Lower jaw deformity (LJD) is a skeletal anomaly affecting farmed triploid Atlantic salmon (*Salmo salar* L.) and leads to considerable economic losses for industry and has animal welfare implications. The present study employed transcriptome analysis in parallel with real-time qPCR techniques to characterise for the first time the LJD condition in triploid Atlantic salmon juveniles using two independent sample sets: experimentally-sourced salmon (60 g) and commercially produced salmon (100 g). A total of eleven genes, some detected/identified through the transcriptome analysis (*fhn2*, *gal* and *gphb5*) and others previously determined to be related to skeletal physiology (*alp*, *bmp4*, *colla1*, *col2a1*, *fgf23*, *igf1*, *mmp13*, *ocn*), were tested in the two independent sample sets. *Gphb5*, a recently discovered hormone, was significantly ($P < 0.05$) down-regulated in LJD affected fish in both sample sets, suggesting a possible hormonal involvement. *In-situ* hybridization detected *gphb5* expression in oral epithelium, teeth and skin of the lower jaw. *Col2a1* showed the same consistent significant ($P < 0.05$) down-regulation in LJD suggesting a possible cartilaginous impairment as a distinctive feature of the condition. Significant ($P < 0.05$) differential expression of other genes found in either one or the other sample set highlighted the possible effect of stage of development or condition progression on transcription and showed that anomalous bone development, likely driven by cartilage impairment, is more evident at larger fish sizes. The present study improved our understanding of LJD suggesting that incorrect development of the cartilage likely underlies the condition and *col2a1* may be a marker. In addition, the involvement of *gphb5* urges further investigation of a hormonal role in LJD and skeletal physiology in general.

4.2 Introduction

Lower jaw deformity (LJD) is a skeletal anomaly affecting the lower jaw of farmed Atlantic salmon (*Salmo salar* L.). Specifically, LJD is a downward curvature of the lower jaw involving dentary and glossohyal bones (Bruno, 1990; Hughes, 1992). LJD has been frequently observed

and identified in both freshwater and seawater phases of production in all countries producing Atlantic salmon at/with different prevalence between years and populations (Benfey, 2001; Sadler *et al.*, 2001; Lijalad and Powell, 2009; Fraser *et al.*, 2013; Taylor *et al.*, 2014; Amoroso *et al.*, 2016a; Fjelldal *et al.*, 2016). Although LJD can occur in diploid populations at very low prevalence, LJD was linked to triploid Atlantic salmon in all recent studies cited above. In Tasmania (Australia) LJD prevalence of up to 30% has been reported in farmed triploid populations (Jungalwalla, 1991; Sadler *et al.*, 2001). Triploids are a valuable part of the annual harvest cycle as they do not undergo sexual maturation therefore can be harvested during the reproductive seasons providing fresh product all year round (Sadler *et al.*, 2001). As a consequence, LJD affected triploid fish represent a considerable loss of production because they have lower growth rates and cannot be sold whole due to their visual unattractiveness (Benfey, 2001; Sadler *et al.*, 2001; Amoroso *et al.*, 2016b). Furthermore, fish affected by skeletal anomalies usually require hand-grading which is an expensive process and adds further cost (Boglione *et al.*, 2013b; Cobcroft and Battaglione, 2013).

Although LJD is a frequently occurring skeletal anomaly in triploid Atlantic salmon its causes have not yet been investigated in depth. Only recently, development of LJD has been linked to dietary phosphorus (P) deficiency and a higher P requirement of triploid Atlantic salmon (Fjelldal *et al.*, 2016). Nevertheless, the mechanisms underlying the onset of LJD are not known and could be multifactorial via a combination of genetic (triploidy and genetic background), nutritional (mineral or vitamin deficiency) and environmental (accelerated growth, low dissolved oxygen, elevated temperature and husbandry practices) factors (Sadler *et al.*, 2001; Lijalad and Powell, 2009; Fraser *et al.*, 2015; Fjelldal *et al.*, 2016). Although it seems to occur mostly during the freshwater phase, onset of LJD can occur at any time in development and prevalence and severity (i.e. worsening of the downward curvature) can

increase over time (Sadler *et al.*, 2001; Fraser *et al.*, 2013; Taylor *et al.*, 2013; Taylor *et al.*, 2014; Fraser *et al.*, 2015; Amoroso *et al.*, 2016a; Amoroso *et al.*, 2016b; Fjelldal *et al.*, 2016).

The lower jaw in Atlantic salmon is a heterogeneous organ constituted of different tissues (e.g. skin, oral epithelium, muscle, adipose tissue, bone, cartilage, thyroid follicles). The bone of the lower jaw (i.e. dentary) is composed of compact bone directly ossifying around the Meckel's cartilage (Hughes, 1992; Sadler, 2000; Witten and Hall, 2002; Gillis *et al.*, 2006). An assessment screening the differential gene expression between LJD and normal individuals, mostly focusing on cartilage and bone physiology, represents a basic approach to shed more light on the mechanisms underlying the condition. Obtaining a significant number of individuals affected by LJD, both in controlled experimental conditions and at commercial farm sites is difficult and labor-intensive. In this research, the opportunity was presented to analyse a sufficient number of fish affected by LJD from independent sample sets and at different developmental stages.

The aims of this study were to delineate for the first time through molecular techniques, transcriptome analysis and real-time qPCR, differential gene expression in the jaw of fish affected by LJD (compared to normal fish) in independent sample sets at different developmental stages and to detect genes which correlate with, and may characterise LJD, allowing the description of possible mechanisms underlying the condition. Furthermore, the specific gene expression pattern observed was used to propose the tissue responsible for the development of the condition. To support the analytical process, our findings are compared at a molecular level with anomalous skeletal processes described in other vertebrates.

4.3 Materials and methods

4.3.1 Sample background, selection and tissue source

Two independent sample sets of all-female triploid Atlantic salmon (*Salmo salar*) individuals were used in this study and both were provided by Petuna Seafoods hatchery in Cressy,

Tasmania. All-female individuals were produced and triploidy was achieved as described in Johnstone *et al.* (1991) and Jungalwalla (1991). Briefly, shock to induce triploidy occurred 30 min after fertilisation when all-female eggs were subjected to a pressure at 9500 psi (655 Bar) for 4 min in water at 10 °C, followed by 1 min for pressure release. Efficiency of the triploid induction was assessed by measuring erythrocyte nuclear length (Benfey *et al.*, 1984) and confirmed as 100% successful. The first sample set (defined “experimental” from now on) was collected at the end of the experiment described in Amoroso *et al.* (2016a). Briefly, the individuals from the experimental sample set were triploidized at hatchery site but reared from incubation up to the sampling event in experimental facilities. Individuals weighing approximately 60 g were sampled in March 2014 following exposure to a standard temperature treatment (14 °C) for two months (additional samples were also collected from individuals exposed to an elevated temperature treatment of 18 °C for two months for additional molecular investigations). The second sample set (defined “industrial” from now on) was reared at a hatchery site and collected in November 2015. Fish sampled weighed approximately 100 g and were subjected to different conditions compared to the experimental sample set (i.e. incubation and rearing temperature) and were derived from different broodstock. For each sample set, fish were euthanized by anaesthetic overdose (AQUI-S: 50 mg L⁻¹) and the lower jaws of fish displaying LJD (LJD $n = 6$ per sample set) (Fig. 4.1a) and of fish displaying a phenotypically normal jaw (Normal $n = 6$ per sample set) (Fig. 4.1a) were dissected and placed in RNA preservation reagent (4 M ammonium sulphate, 25 mM sodium citrate, 10 mM EDTA; pH 5.2) to preserve RNA integrity. The samples were held at 4 °C overnight and stored at – 20 °C for a maximum of two months before processing for molecular analysis. For histological analysis, the lower jaws of individuals from the experimental sample set displaying LJD ($n = 3$) and phenotypically normal jaw ($n = 3$) were dissected and placed in Bouin’s solution overnight and then preserved in 70% ethanol for a maximum of four months before the analysis. All

procedures were carried out with the approval of the University of Tasmania Animal Ethics Committee (approval number A0013044).

4.3.2 RNA extraction and preparation for next generation sequencing

The lower jaw samples from the sample sets described above were carefully dissected, removing excess tissues in order to leave dentary bone and a thin layer of the surrounding tissues. Samples were then homogenized using a LabGEN 7 Series Homogenizer (Cole Parmer, Vernon Hills, IL, USA) in vials containing RNazol® RT (Molecular Research Centre Inc., Cincinnati, OH, USA) for the isolation of total RNA, following manufacturer's instructions. The isolated RNA was tested for quality and quantity using a NanoDrop 2000 spectrophotometer (Thermo Scientific, NanoDrop Products, Wilmington, DE, USA). For next generation sequencing, fish from the experimental sample set affected by LJD ($n = 6$) and phenotypically normal (Normal) ($n = 6$) were used. Equal amounts of RNA from three individuals from the same category were mixed to generate a total of four pooled samples representing two replicates of LJD and two replicates of Normal. The Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) was used to validate quantity and quality of RNA. All samples had RIN (RNA Integrity Number) values higher than 7. The qPCR validation was done on the six original biological replicates, not on the pooled samples.

4.3.3 Next generation sequencing and data handling

Samples were prepared for sequencing by the Australian Genome Research Facility (AGRF, Melbourne, Australia) according to the manufacturer's instructions (Illumina, San Diego, CA, USA). Briefly, poly (A) mRNA was isolated using oligo (dT) beads and the addition of fragmentation buffer for shearing mRNA into short fragments (200 – 700 nt) prevented priming bias during the synthesis of cDNA using random hexamer-primers. The short fragments were further purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany) and resolved

with EB buffer for ligation with Illumina Paired-end adapters. This was followed by size selection (~200 bp), PCR amplification and Illumina sequencing using an Illumina Genome Analyzer (HighSeq 2000, Illumina, San Diego, CA, USA) performing 100 bp-paired end sequencing. The sequence reads were stored as FASTQ files. Overall, at least 4 Gb of cleaned data (at least 50 million reads) was generated for each of the four samples sequenced. Prior to assembly, quality of the FASTQ files was assessed using CLC Genomics Workbench v4 (CLC bio, Aarhus, Denmark), using default parameters. Based on the QC reports, FASTQ files were trimmed using CLC with default parameters with the addition of trimming 10 nucleotides from the 5' of all reads.

4.3.4 Bioinformatics and statistics

De novo assembly of the trimmed reads was performed in CLC Genomics Workbench v4 using default parameters with the exception of minimum contig length elevated to 500. Trimmed reads were mapped to the assembly in CLC Genomics Workbench v4 using default parameters with the exception of similarity fraction elevated to 0.9. BAM files (resulting in $77.05 \pm 0.23\%$ mapped reads per library) were then imported into Partek Genomics Suite (Partek Incorporated, St. Louis, MO, USA) for differential gene expression (DGE) analysis. In Partek GS, categorical attributes were assigned to each duplicate in the LJD and Normal, followed by DGE analysis without restricting paired-end compatibility. One-way ANOVA was performed in Partek GS to compare reads per kilobase per million (RPKM) with contrast between the LJD and Normal samples. The same one-way ANOVA procedure was performed following restricting RPKM ≥ 1 in at least one sample and defining the minimum as RPKM = 0.05. The final list of transcripts used in the analysis was retrieved by selecting transcripts having at least a 2 fold change between the LJD and Normal samples with an unadjusted significance level of $P < 0.05$.

The prediction of the amino-acid sequences corresponding to the transcripts was performed using the ORF-PREDICTOR website

(<http://proteomics.ysu.edu/tools/OrfPredictor.html>). CLC main workbench 7.5 (CLC Inc, Aarhus, Denmark) at default parameters was used to perform a BLASTP (sequence comparisons and alignment) against the database of the National Center for Biotechnology Information (NCBI) and annotate and predict the most likely corresponding product (best hit) in Atlantic salmon. After the BLASTP, only matches with E-value $\leq 1.00\text{E-}40$ were selected for the analysis. Discrimination of the best hit obtained for each sequence was performed based on the E-value $\leq 1.00\text{E-}40$. In case the first best hit resulted in an unannotated product the second was chosen (named 'actual hit'). In order to ascertain that the expression of the differentially expressed genes was not basal, only the subset of transcripts where the sum of RPKM in all four samples was ≥ 5 , were considered.

4.3.5 cDNA synthesis, probe and primer design for qPCR

Parallel to the transcriptome analysis, we tested in Normal and LJD samples from the experimental sample set a group of previously known transcripts whose function was annotated to be related to bone and cartilage physiology in vertebrates. The transcripts selected were: *alkaline phosphatase (alp)*, *bone morphogenetic protein 4 (bmp4)*, *collagen type I alpha 1 (colla1)*, *collagen type II alpha 1 (col2a1)*, *fibroblast growth factor 23 (fgf23)*, *insulin like growth factor 1 (igf1)*, *matrix metalloproteinase 13 (mmp13)* and *osteocalcin (ocn)* (Table 4.1). Among the transcripts found to be differentially expressed after transcriptome analysis, the following were selected according to the quantitative values of differential expression, concurrently with their previously known or possible relation to bone and cartilage physiology: *fibrillin 2 (fbn2)*, *galactose-specific lectin (gal)* and *glycoprotein hormone beta 5 (gphb5)* (Table 4.2). All the eleven transcripts reported above were later tested in Normal and LJD samples from the industrial sample set to compare gene expression patterns between the independent sample sets. Furthermore, some of the transcripts (*alp*, *colla1*, *col2a1*, *mmp13* and *ocn*) were also tested in Normal and LJD samples from the additional sample set (elevated

rearing temperature) in order to investigate the possible effect of the temperature on differential expression (14 °C vs 18 °C). 18S only served as a housekeeping calibrator gene in both assays as showing to be very stable (Ct range: 12-13 experimental group and 17-19 industrial group). Following RNA extraction and quantification, 1 µg of total RNA was reverse-transcribed into cDNA using Tetro cDNA synthesis kit (Bioline, London, UK), according to manufacturer's instructions. RNA samples were not DNase treated prior to cDNA synthesis. Probes for qPCR were designed by the Universal ProbeLibrary System (Roche, <http://www.roche-applied-science.com>) and primers for transcripts previously annotated were purchased from GeneWorks Pty Ltd (Hindmarsh, SA, Australia) while primers for transcripts selected after transcriptome analysis were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Primer quality testing was not carried out as not necessary when using the Universal ProbeLibrary. The Roche software provides the optimal primer parameters and, given the ultra-short amplicon size (60-72 nucleotides), the chance of secondary amplicons is negligible.

4.3.6 Real-time qPCR assays

Real-time qPCR assays were performed in duplicates on each of the biological replicates ($n = 6$ per group) using FastStart Universal Probe Master (ROX) (Roche, Australia) according to manufacturer's protocol in a Rotor-Gene 6000 Real-Time PCR Machine (Corbett Robotics Pty Ltd, Brisbane, Australia) with the following thermal cycling conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 60 sec. For each gene the control used was a duplicate no-template. Average cycle threshold for each duplicate was calibrated relative to 18S and basal expression levels (which refers to the lowest expressing tissue measured) and transformed to represent relative expression quantity as $2^{-\Delta\Delta CT}$. Nonparametric test for independent samples (Mann-Whitney U test) was used to investigate significant differences between relative expression levels of each transcript between traits (Normal and LJD) and different temperature treatments. All data analyses were performed using GraphPad Prism

version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA) with a significance level of $P < 0.05$. Results are represented as mean \pm standard error of the mean (SEM).

Table 4.1. Primers and probes used for real-time qPCR designed from previously known transcripts whose function was annotated to be related to bone and cartilage physiology (abbreviations described in the text).

Transcript	Orientation	Genbank accession number	Tm	Sequence (5'-3')	Probe cat.no. (Roche)
<i>alp</i>	Forward Reverse	FJ195609.1	59 59	cagctgagcagacagagtgg caacaaaggggaactgtcc	04689011001
<i>bmp4</i>	Forward Reverse	NM_001139844.1	60 60	ggttgccgctaactgact tggggtcttttcttagcgtct	04687582001
<i>col1a1</i>	Forward Reverse	FJ195608.1	60 59	agcctgggtgctaagggagag ccttagctccggtgtttcc	04688619001
<i>col2a1</i>	Forward Reverse	FJ195613.1	59 59	tcgacatgtctgccttcg tcagccctcatgtacctcaa	04693442001
<i>fgf23</i>	Forward Reverse	Sequence from our sequencing database	59 60	ggatcagaagggtcaaccac aacacggtgccactggag	04685059001
<i>igf1</i>	Forward Reverse	EF432852.2	60 59	ggcttttatttcagtaaaccaac cgtccacaataaccacggta	04688546001
<i>mmp13</i>	Forward Reverse	NM_001140524.1	59 60	ccattccctcgggtctcag agggtgctggggtttgtgtag	04684974001
<i>ocn</i>	Forward Reverse	FJ172977.1	60 60	tgtgtgtgccactctattgga tctttctcactagcaggcttg	04689119001
<i>18s</i>	Forward Reverse	FJ710886.1	59 60	aggactccggttctattttgtg cggccgtccctctaatac	04688546001

Table 4.2. Primers and probes used for real-time qPCR from selected transcripts found differentially expressed after transcriptome analysis in the experimental sample set (abbreviations described in the text).

Transcript	Orientation	Tm	Sequence (5'-3')	Probe cat.no. (Roche)
<i>fbn2</i>	Forward Reverse	59 60	cacgacagcgacacttgaa ggctcacaactgtgacatgc	04687582001
<i>gal</i>	Forward Reverse	59 59	ctttgaactgcagtgagaccac tcatgactcccatgatgacc	04694449001
<i>gphb5</i>	Forward Reverse	60 59	tgtaggaggagggtcaaggaca gagggttcacatcaccac	04685059001

4.3.7 General Histology

The left halves of the lower jaws collected from the experimental sample set were used for all histological analyses. Tissues were rinsed and decalcified for 72 h in a 10% EDTA solution buffered with 0.1 M TRIS base, pH 7.0 as described in Witten and Hall (2003) and supplemented with ProtectRNA™ RNase Inhibitor (Sigma-Aldrich, Castle Hill, NSW, Australia). After that, a protocol modified from Ventura *et al.* (2011) was used. Briefly, tissues were dehydrated gradually through a series of increasing alcohol concentrations and embedded in Paraplast® Plus (McCormick Scientific Leica™, North Ryde, NSW, Australia) according to conventional procedures. Serial sections of 7 µm were cut from the sagittal plane of the lower jaw, until reaching the area in which teeth, bone and Meckel's cartilage were visible, and placed onto Superfrost™ Ultra Plus Adhesion Slides (Thermo Fisher Scientific, Scoresby, VIC, Australia). Duplicate and consecutive sections were used for Hematoxylin and Eosin (H&E) staining and *in-situ* hybridization.

4.3.8 H&E staining

The slides were deparaffinized in xylene and rehydrated gradually through a series of decreasing alcohol concentrations (100%, 90%, 70%, 50%). After rinsing in water the slides were stained in hematoxylin for 4 min and rinsed again. Slides were placed for 30 sec in acidic alcohol (70% + 0.1% HCl), rinsed, stained in eosin for 3 min, rinsed again and gradually dehydrated through a series of increasing alcohol concentrations (50%, 70%, 90%, 100%), bathed in xylene and finally mounted.

4.3.9 In-situ Hybridization

To design the primers for the *in-situ* hybridization probes, the sequences obtained from the transcriptome analysis of *gphb5* were blasted using Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and primers (Sequence 5'-3' - Forward gtgtacatggggtccacgtt and Reverse gagaagcctgtccttgacct) purchased from Sigma-Aldrich

(Castle Hill, NSW, Australia). Digoxigenin-labeled oligonucleotides for antisense and sense probes were synthesized using T7 RNA polymerase, and the probes were hydrolysed to reduce their length to approximately 200 bases, as described in the Digoxigenin Application Manual (Roche Applied Science, Indianapolis, IN). Slides with samples from the lower jaw were deparaffinised, rehydrated, rinsed in diethyl pyrocarbonate (DEPC)-treated water and then washed in PBS for 6 min. Samples were digested with 5 $\mu\text{g ml}^{-1}$ of proteinase K (Roche Diagnostics GmbH) in PBS with Tween 20 (PBST) for 10 min at 37 °C and incubated in PBST containing 2 mg ml^{-1} of glycine for 5 min at room temperature. Samples were rinsed again two times in PBST and fixed in 4% paraformaldehyde in PBS (10 mM phosphate buffer Na_2HPO_4 , 150 mM NaCl, pH 7.4) for 4 min and again washed PBS containing 0.1% DEPC for 20 min and in PBS until pre-hybridization at room temperature. Pre-hybridization was performed at 48 °C for 2 h in pre-hybridization buffer (50% 20X formamide, 10% saline sodium citrate, 40% dextran sulfate, tRNA 10 mg/ml, heparin 50 mg/ml and 10 mg ml^{-1} of sheared and denatured salmon sperm DNA). Hybridization was performed at 48 °C overnight with 0.2 $\mu\text{g ml}^{-1}$ of antisense and sense probes in hybridization buffer (identical to pre-hybridization buffer). Samples were washed three times for 5 min in 4X wash (50% formamide, 30% DEPC-treated water, 20% 20X SSC - 0.15 M sodium chloride and 0.015 M sodium citrate - 50 μl of Tween 20), three times for 5 min in 2X wash (50% formamide, 40% DEPC-treated water, 10% 20X SSC, 50 μl of Tween 20) and three times for 5 min in 1X wash (50% formamide, 45% DEPC-treated water, 5% 20X SSC, 50 μl of Tween 20) at 48 °C. Samples were washed again three times for 5 min in 1X SSC with 0.1% Tween 20 and two times for 2 min in maleic acid buffer (MAB) (0.1 M maleic acid, 0.015 M NaCl, 0.1% Tween 20, pH 7.5) at room temperature. Blocking was performed at room temperature for 2 h with MAB block (2% BM block in MAB) and finally samples were incubated with Anti-Digoxigenin-AP (Roche, Australia) at 4 °C overnight. Following incubation, samples were washed four times for 5 min with MAB, two

times for 5 min with 1X alkaline phosphatase buffer (AP) (50% 1 M Tris, 40% DEPC-treated water, 10% 5 M NaCl, 0.01% Tween 20) and two times for 5 min 1X AP with 5% MgCl₂ at room temperature. Final incubation was performed at 4 °C overnight with 20 µl ml⁻¹ of NBT/BCIP (Roche, Australia) in developmental buffer (5% polyvinyl alcohol in 1X AP with 5% MgCl₂). Slides were dehydrated in alcohol 70%, 100% and cleared with xylene before mounting with DPX (Sigma-Aldrich). Sections were observed under a Nikon ECLIPSE E600 light microscope and photographed.

4.4 Results

4.4.1 Transcriptome analysis of the experimental sample set

De novo assembly of the transcriptome data gave a total of 62,373 contigs (including scaffolded regions) with a minimum length of 500, a maximum of 14,769 and an average of 1,482 bases.

A total of 515 transcripts had at least 2 fold change between the LJD and Normal samples (unadjusted $P < 0.05$; RPKM ≥ 1 in at least one sample). When the RPKMs were modified to a minimum of 0.05, a total of 6,207 transcripts had at least 2 fold change between the LJD and Normal samples (unadjusted $P < 0.05$; RPKM ≥ 1 in at least one sample), indicating that most of the DGE can be attributed to transcripts which do not express in one group while they do in the other. Since in most cases these transcripts had very small RPKM values, we decided to focus on the 515 transcripts.

Out of the 515 transcripts, 452 were down-regulated and 63 were up-regulated in LJD. The hierarchical clustering of differential gene expression showed that samples clustered tightly together based on jaw trait (LJD/Normal; Fig. 4.1b left), strengthening the validity of the differential gene expression analysis.

Following BLASTP, selection according to E-value (for matches and hits) and RPKM-based filter (as described in the materials and methods section), two tables were produced: one consisting of 6 transcripts down-regulated in LJD samples (Table 4.3) and another consisting

of 27 transcripts up-regulated in LJD samples (Table 4.4) ordered by E-value of the best hit (lowest to highest).

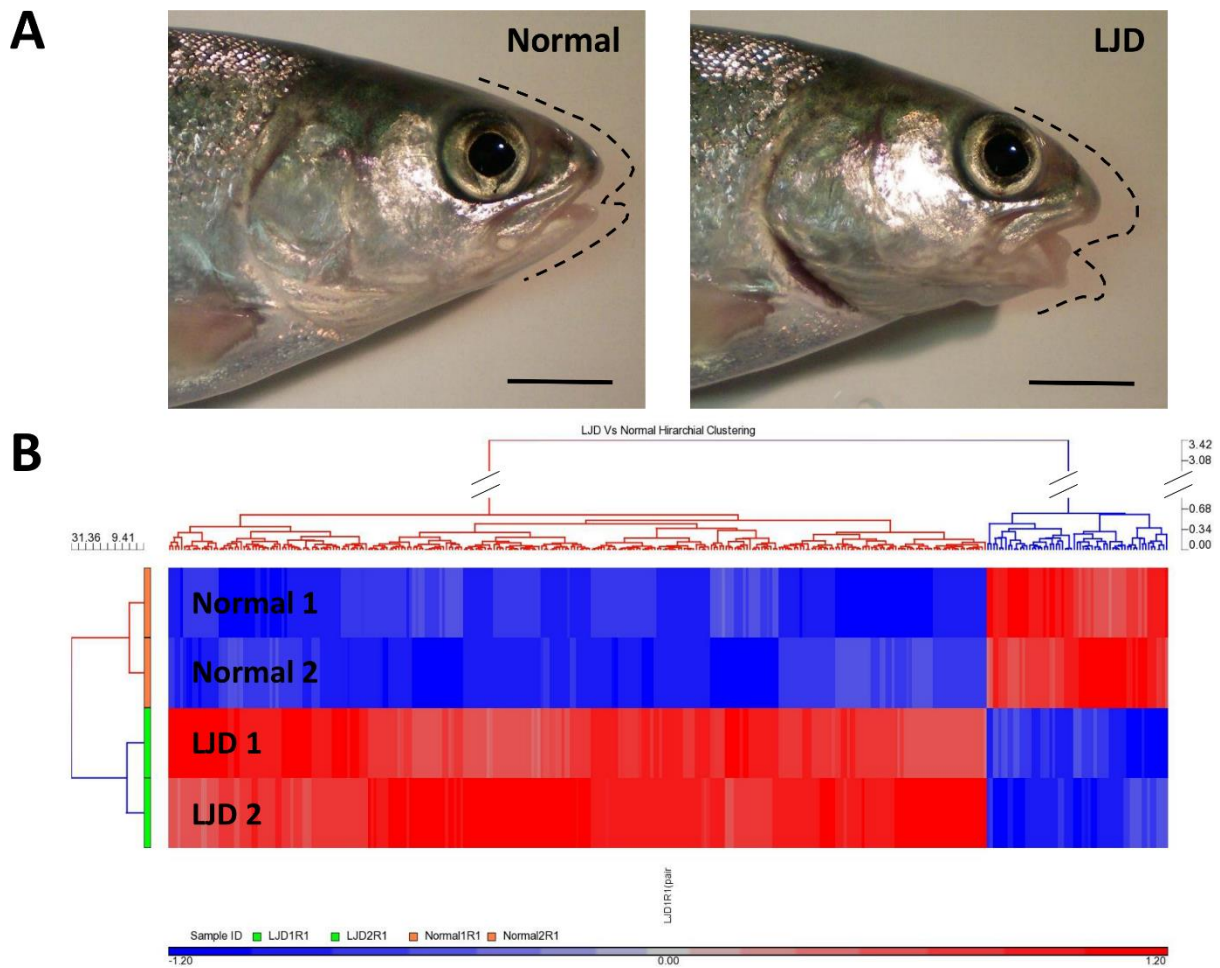


Fig. 4.1. a) Atlantic salmon individuals displaying a normal jaw (Normal) on the left and a lower jaw deformity (LJD) on the right (scale bar = 1 cm). Dashed lines highlight normal and anomalous profiles. **b)** Hierarchical clustering showing differentially expressed transcripts between Normal vs. LJD grouped samples retrieved after a pairwise comparison. Each row corresponds to one sample tested and each column corresponds to a single differentially expressed transcript. On the hierarchical tree at the left side of the map, the upper half (orange) indicates the Normal samples and the lower half (green) indicates the LJD samples. Relative gene expression is indicated by colour: red is higher-level expression relative to the sample mean, blue is relatively lower-level expression, grey is no-change. The 452 genes on the left were up-regulated in LJD samples relative to the Normal samples. The 63 genes on the right were down-regulated in LJD samples relative to the Normal samples.

4.4.2 Down-regulated transcripts

Among the 63 transcripts of this subset, 18 could be reliably annotated via NCBI database with E-value $\leq 1.00\text{E-}40$. Of the 18 transcripts, six had a Sum of RPKM ≥ 5 across all four pooled samples (Table 4.3). Transcript 59181, which corresponded to glycoprotein hormone beta 5

(*gphb5*) had a much higher fold change (-4.3) between the groups compared to other transcripts. For five out of the above six transcripts, the best hit resulted in an unannotated product described for *Oncorhynchus mykiss* (rainbow trout), a closely-related Salmonid species. The actual hit for these transcripts resulted in products described in other Teleost species (Table 4.3). Transcript 30200, which corresponded to aggrecan isoform 1 (*acan1*) and that was annotated as aggrecan, had a best hit with a named product in the Salmonid *Oncorhynchus keta* (Chum salmon) (Table 4.3).

Table 4.3. The six transcripts found down-regulated in LJD selected according to Sum of RPKM ≥ 5 across all 4 pooled samples ordered by E-value of the best hit (lowest to highest).

Transcript	Accession Best Hit	E-value Best hit	Accession Actual Hit	Predicted product [species]	E-value Actual Hit
30200	BAJ61837	0.00	BAJ61837	aggrecan [<i>Oncorhynchus keta</i>]	0.00
8151	CDQ60370	0.00	BAJ61837	aggrecan [<i>Oncorhynchus keta</i>]	0.00
15298	CDQ84852	1.61E-137	XP_005945737	cytosolic phospholipase A2 gamma-like isoform X2 [<i>Haplochromis burtoni</i>]	1.24E-89
59181	CDQ81732	7.53E-96	XP_005455982	glycoprotein hormone beta-5-like [<i>Oreochromis niloticus</i>]	2.20E-72
662	CDQ80483	5.85E-86	XP_007243147	protein FAM111A-like [<i>Astyanax mexicanus</i>]	6.29E-75
26682	CDQ61726	5.09E-82	XP_007242859	mannose-specific lectin-like [<i>Astyanax mexicanus</i>]	1.23E-62

4.4.3 Up-regulated transcripts

Among the 452 transcripts of this subset, 176 could be reliably annotated via NCBI database with E-value $\leq 1.00\text{E-}40$. Of the 176 transcripts, 27 had a Sum of RPKM ≥ 5 (Table 4.4). Transcript 40854 was annotated as Four and a half LIM domains protein 2, transcript 2193 corresponded to galactose-specific lectin (*gal*) and transcript 45908 was annotated as major histocompatibility complex (MHC) class I antigen. These transcripts had a much higher fold change (3.2, 3.5 and 4.6, respectively) between the groups, as compared with other transcripts. For 15 out of the 27 transcripts, the best hit resulted in an unannotated product described for

the species *O. mykiss*. The actual hit for these transcripts resulted in products described in other Teleost species (Table 4.4). Seven of the remaining 12 transcripts had best hits with named products described for *S. salar*, and the last five with other Teleost species.

Table 4.4. The 27 transcripts found up-regulated in LJD selected according to Sum of RPKM ≥ 5 across all 4 pooled samples ordered by E-value of the best hit (lowest to highest). *Continued on the next page.*

Transcript	Accession Best Hit	E-value Best Hit	Accession Actual Hit	Predicted product [species]	E-value Actual Hit
3859	CDQ85625	0.00	XP_006641632	IgGFc-binding protein-like [<i>Lepisosteus oculatus</i>]	5.60E-152
718	CDQ93202	0.00	XP_006641633	IgGFc-binding protein-like [<i>Lepisosteus oculatus</i>]	0.00
407	CDQ85625	0.00	XP_006641632	IgGFc-binding protein-like [<i>Lepisosteus oculatus</i>]	1.21E-151
5789	XP_005450984	0.00	XP_005450984	filamin-C-like isoform X3 [<i>Oreochromis niloticus</i>]	0.00
1330	CDQ86144	0.00	XP_008284200	xin actin-binding repeat-containing protein 1-like [<i>Stegastes partitus</i>]	0.00
10204	XP_007252649	0.00	XP_007252649	collagen alpha-2(VI) chain-like [<i>Astyanax mexicanus</i>]	0.00
894	XP_006808726	0.00	XP_006808726	von Willebrand factor A domain-containing protein 7-like [<i>Neolamprologus brichardi</i>]	0.00
40854	ACI68280	0.00	ACI68280	Four and a half LIM domains protein 2 [<i>Salmo salar</i>]	0.00
26217	ADD59862	0.00	ADD59862	immunoglobulin delta heavy chain constant region [<i>Salmo salar</i>]	0.00
31731	CDQ69338	0.00	XP_005163674	amylo-1, 6-glucosidase, 4-alpha-glucanotransferase isoform X1 [<i>Danio rerio</i>]	2.68E-128
7575	CDQ73728	4.69E-171	XP_008279287	pecanex-like protein 3 [<i>Stegastes partitus</i>]	5.79E-171
22520	CDQ71312	7.88E-158	XP_008293195	eukaryotic translation initiation factor 2-alpha kinase 4 [<i>Stegastes partitus</i>]	6.99E-120
12561	CDQ83306	3.64E-156	XP_006641633	IgGFc-binding protein-like [<i>Lepisosteus oculatus</i>]	1.42E-113
41692	CDQ61095	7.87E-156	XP_003966210	serum amyloid P-component-like [<i>Takifugu rubripes</i>]	2.85E-81
5264	ACI68585	2.30E-154	ACI68585	Heat shock protein 30 [<i>Salmo salar</i>]	2.30E-154
15329	NP_001118064	2.22E-135	NP_001118064	heat shock protein, alpha-crystallin-related, 1 [<i>Oncorhynchus mykiss</i>]	2.22E-135
44416	CDQ81901	3.22E-125	XP_007243338	protein NLRC3-like [<i>Astyanax mexicanus</i>]	1.85E-70

15155	CDQ66610	7.50E-124	XP_008292526	protein NLRC3-like [<i>Stegastes partitus</i>]	9.50E-121
27480	CDQ68760	3.73E-113	XP_008278793	sortilin-related receptor [<i>Stegastes partitus</i>]	1.33E-84
2193	CDQ61022	2.60E-108	ACO13356	Galactose-specific lectin [<i>Esox lucius</i>]	5.21E-09
35957	XP_007258003	3.18E-100	XP_007258003	ryanodine receptor 3-like [<i>Astyanax mexicanus</i>]	3.18E-100
6273	CDQ74429	1.20E-84	XP_008303180	fibrillin-2-like, partial [<i>Stegastes partitus</i>]	1.35E-82
1876	NP_001117045	2.06E-83	NP_001117045	cathelicidin antimicrobial peptide precursor [<i>Salmo salar</i>]	2.06E-83
10290	NP_001134766	3.32E-61	NP_001134766	Heat shock protein beta-7 [<i>Salmo salar</i>]	3.32E-61
25466	NP_001134309	3.03E-59	NP_001134309	Natterin-like protein [<i>Salmo salar</i>]	3.03E-59
34091	CDQ58999	2.04E-56	XP_003968600	serine/threonine-protein kinase Nek8-like [<i>Takifugu rubripes</i>]	3.05E-47
45908	ACY30362	6.08E-48	ACY30362	MHC class I antigen [<i>Salmo salar</i>]	6.08E-48

4.4.4 Real-time qPCR validation

The differential expression observed after the transcriptome analysis was confirmed for three out of five transcripts. In particular, fibrillin 2 (*fbn2*) and *gal* were significantly ($P < 0.05$) up-regulated in LJD affected fish while *gphb5* was significantly ($P < 0.05$) down-regulated in LJD affected fish (Fig. 4.2). The differential expression of both *acan1* and *acan2*, that were significantly down-regulated in LJD affected fish according to the transcriptome analysis, was not confirmed by real-time qPCR assays (Fig. 4.2).

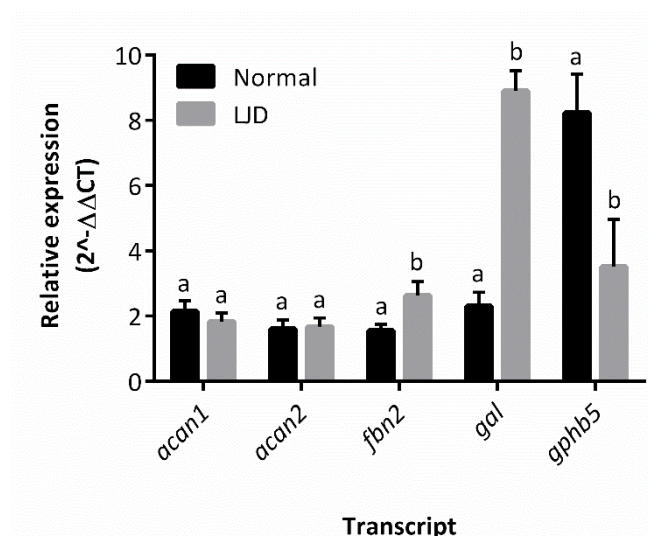


Fig. 4.2. Relative expression ($2^{-\Delta\Delta CT}$, mean \pm SEM) of selected transcripts (abbreviations described in the text) found to be differentially expressed in two different categories (Normal and affected by lower jaw deformity, LJD) after transcriptome analysis of triploid Atlantic salmon *Salmo salar* pre-smolts from the experimental sample set ($n = 6$ per jaw trait). For each transcript, expression level means were compared between different categories. Within each transcript, means significantly different from one another are indicated by different letters ($P < 0.05$).

4.4.5 Real-time qPCR of different developmental stages

Testing all the eleven transcripts available after initial selection and following transcriptome analysis in both the experimental and the industrial sample set, only *col2a1* and *gphb5* showed the same regulation pattern in both independent sample sets (Fig. 4.3). In particular, *col2a1* and *gphb5* were significantly ($P < 0.05$) down-regulated in fish with LJD compared to Normal fish in the two independent sample sets (Fig. 4.3). Other transcripts were found to be differentially expressed between traits in one sample set only. In particular, *fbn2* and *gal* were significantly ($P < 0.05$) up-regulated in LJD in the experimental sample set only while *alp*, *bmp4*, *col1a1*, *igf1* and *mmp13* were significantly ($P < 0.05$) down-regulated in LJD in the industrial sample set only (Fig. 4.3). *Fgf23* and *ocn*, showed no differential expression between traits in both sample sets.

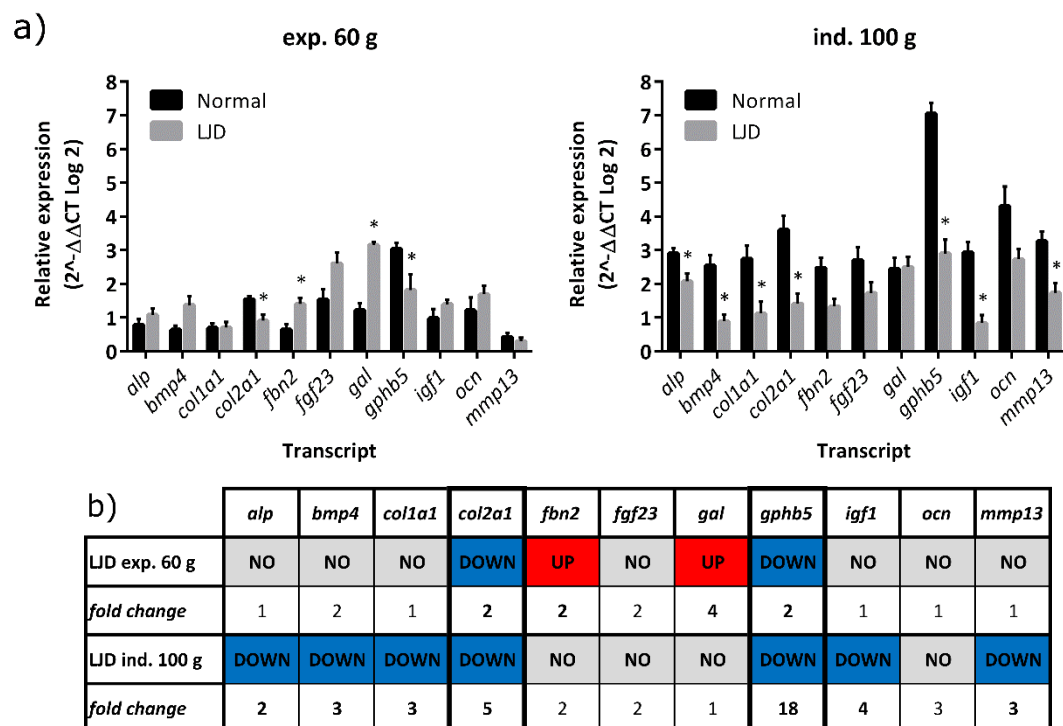


Fig. 4.3. a) Relative expression ($2^{-\Delta\Delta CT}$, mean + SEM Log 2) of the eleven transcripts tested in triploid Atlantic salmon *Salmo salar* individuals belonging to two independent sample sets, ‘experimental’ (exp. 60 g) on the left and ‘industrial’ (ind. 100 g) on the right, and displaying a normal lower jaw (Normal) or a lower jaw deformity (LJD) ($n = 6$ per jaw trait). Significantly different ($P < 0.05$) relative expression between traits is indicated by an asterisk. **b)** A graphic summary of the previous graphs to show significantly different regulation (UP - red, DOWN - blue, NO - grey) and corresponding approximate fold change (significant in bold) in LJD individuals only relative to Normal from the two independent sample sets. Columns of *col2a1* and *gphb5* have thicker borders to highlight consistent differential expression between independent sample sets.

4.4.6 Real-time qPCR for fish reared at different temperatures (14 vs 18 °C)

Among the transcripts tested for the effect of rearing temperature only *col2a1* showed to be differentially expressed. In particular, *col2a1* was significantly ($P < 0.05$) down-regulated in LJD within both temperature treatments and significantly ($P < 0.05$) up-regulated in both traits in the elevated treatment (18 °C) compared to the standard treatment (14 °C) (Fig. 4.4).

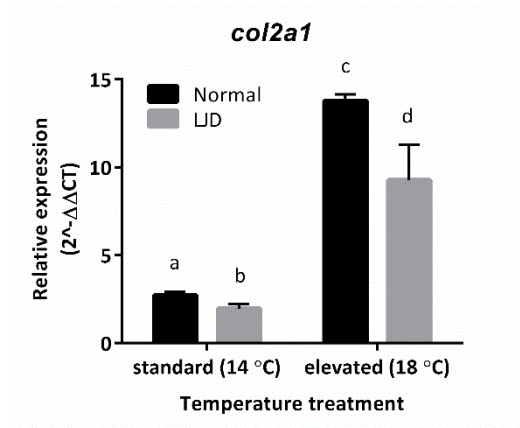


Fig. 4.4. Relative expression ($2^{-\Delta\Delta CT}$, mean \pm SEM) of *col2a1* in Normal and lower jaw deformity (LJD) affected triploid Atlantic salmon *Salmo salar* ($n = 6$ per jaw trait) from the experimental sample set at standard (14 °C) and elevated (18 °C) temperature. Means significantly different from one another are indicated by different letters ($P < 0.05$).

4.4.7 GPHB5 In-Situ Hybridization

In-situ hybridization analysis showed the expression of *gphb5* in both traits (Normal and LJD) to be mostly in the skin and more evident in the oral epithelium and at the tip of the lower jaw (Fig. 4.5). Furthermore, *gphb5* was also expressed around the teeth, in particular in the outer dental epithelium and around the dental papilla (Fig. 4.5). Given the general low expression of the gene a quantitative differential expression between Normal and LJD was not observed.

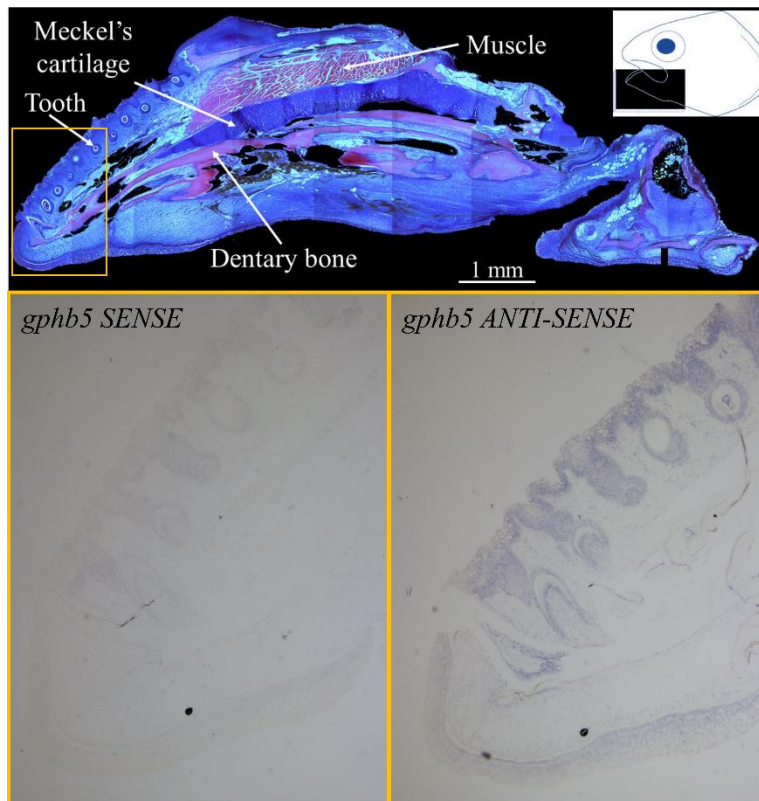


Fig. 4.5. Top panel: a transverse H&E stained section of the lower jaw of a triploid Atlantic salmon (~60 g) affected by lower jaw deformity (LJD). Image series was tiled and black background was added using Photoshop. White arrows indicate the main anatomical features. In the top right corner, the drawing illustrates the location and position of the sections obtained. Bottom panels: *in-situ* hybridization stained sections of a LJD affected jaw (region magnified from the corresponding H&E staining image is outlined in yellow at the top panel). Bottom left panel shows the negative control where *gphb5* sense probe was used while in c) the bottom right the tissue was hybridizing with the *gphb5* anti-sense probe. In the latter, the signal is detectable (purple stain) in the oral epithelium, around the teeth and in the skin at the tip of the jaw.

4.5 Discussion

In the present study, among the eleven genes tested in individuals displaying a normal lower jaw or a LJD, only *col2a1* and *gphb5* showed the same consistent pattern of differential expression, being down-regulated in LJD affected fish in both independent sample sets. This result implies that, in contrast to other genes found differentially expressed between traits either in one sample set or in another, *col2a1* and *gphb5* are reliable indicators of the mechanisms underlying LJD.

Col2a1 is an exclusive marker of cartilage and is responsible for the expression of type II collagen, a basic protein for skeletogenesis in vertebrates (Cheah *et al.*, 1985; Cheah *et al.*, 1991; Erlebacher *et al.*, 1995; Karsenty *et al.*, 2009). In the present study, down-regulation of *col2a1* in LJD affected fish may thus indicate a compromised development of the Meckel's cartilage through loss of structural integrity and incorrect growth trajectory (pointing downward) of the deriving or integrally-linked bone structure, the dentary bone (main lower

jaw bone). Type II collagen is produced by chondrocytes and represents the most abundant protein in the cartilage extracellular matrix and is crucial for cartilage conformation and resistance (Stockwell, 1979; Upholt, 1989; Erlebacher *et al.*, 1995; Karsenty *et al.*, 2009). In Atlantic salmon, Meckel's cartilage does not ossify and plays a crucial role in physically supporting the dentary, extending internally almost for its entire length (Sadler, 2000; Witten and Hall, 2002; Gillis *et al.*, 2006). Considering the consistency of down-regulation of *col2a1* in LJD-affected fish between independent sample sets from different developmental stages (60 vs 100 g), this finding suggests a cartilaginous impairment as a possible distinctive feature of the trait.

Defects in type II collagen have frequently been associated with the occurrence of impairments in cartilage and as a consequence bone development in humans and mice (Murray *et al.*, 1989; Tiller *et al.*, 1990; Garofalo *et al.*, 1991; Vikkula *et al.*, 1994; Li *et al.*, 1995; Kuivaniemi *et al.*, 1997; Prockop *et al.*, 1997; Rani *et al.*, 1999; Donahue *et al.*, 2003; Sahlman *et al.*, 2004; Savontaus *et al.*, 2004; Richards *et al.*, 2010). In support of our results, disruption of cartilage growth and development in LJD has been reported by previous studies. X-ray analysis showed a diminution of the Meckel's cartilage in LJD-affected fish (Bruno, 1990) and histological examination of LJD-affected Meckel's cartilage showed different left to right thickness, implying an impaired development (Hughes, 1992). Likewise, the lower jaw of LJD-affected fish presented an incorrect assemblage and a smaller number of collagen fibres, adding to the evidence that the anomaly may be due to an impairment in the jaw cartilage development (Venegas *et al.*, 2003). The studies mentioned above, analysed later stages of fish development compared to the current study, supporting our theory that cartilaginous impairment is a distinctive feature of LJD at any stage.

In the present study, the effect of water temperature on the up-regulation of *col2a1* in both jaw traits may be explained by an enhanced sensitivity of chondrocytes to higher

temperatures (i.e. change in shape of chondrocytes) as shown for vertebral deformities in Atlantic salmon exposed to high water temperature (Ytteborg *et al.*, 2010b). In light of the above, prolonged exposure to high water temperature, in particular for Atlantic salmon above 14-15 °C, may lead to a possible exacerbation of cartilage impairment and likely increased LJD severity in both originally normal and LJD affected fish, but with likely greater impact on the latter.

The consistent down-regulation of *gphb5* in LJD affected fish in both independent sample sets, as well as its localization through *in-situ* hybridization in this study, provides further insight into the onset of the condition, pointing towards a possible hormonal involvement. *Gphb5* is an evolutionarily well conserved glycoprotein hormone described for the first time in humans in 2002 (Hsu *et al.*, 2002). Expression of *gphb5* occurs in several species in the pituitary, implying a role in hypothalamus-pituitary peripheral tissue (HPT) axis (Hsu *et al.*, 2002; Nakabayashi *et al.*, 2002). *Gphb5* is known to activate the thyroid-stimulating hormone (TSH) receptors in cells of thyroid and it was named thyrostimulin due to its ability to stimulate thyrotropin receptors (Nakabayashi *et al.*, 2002). Nevertheless, the primary role of the hormone is still unknown (Bassett *et al.*, 2015). *Gphb5* seems to have a paracrine rather than an endocrine function, implying that it can be expressed locally and induce changes in nearby cells (Nakabayashi *et al.*, 2002; Bassett *et al.*, 2015). In the present study, the expression of *gphb5* in the skin cells is in accordance to that found in humans (Bodó *et al.*, 2010). Furthermore, it has been recently shown that *gphb5* plays a paracrine role in skeletal development and bone formation (Bassett *et al.*, 2015). In light of the above, our results may suggest that *gphb5* is expressed in the skin of the lower jaw and induce changes in cartilage or bone cells located at short distance. Furthermore, considering the aforementioned relation *gphb5*-thyroid, *gphb5* down-regulation and consequent lower expression in LJD-affected individuals may indicate an underactive thyroid. Underactive thyroid is generally linked with

impairments in chondrocyte differentiation and linear growth as well as bone formation and mineralisation (Bassett *et al.*, 2015). Since, as suggested by our results, LJD could be the result of a cartilaginous impairment with resulting deleterious effects on bone development, the findings reported above support and reinforce our theory that *gphb5* is likely involved in mechanisms underlying LJD and, although not expressed specifically in cartilage or bone in the present study (mostly in oral epithelium, teeth and skin), *gphb5* may act through paracrine pathways. The literature for this relatively novel gene is limited and further investigations are needed to verify its role in skeletal development and HPT (Nakabayashi *et al.*, 2002; Bodó *et al.*, 2010; Bassett *et al.*, 2015). Nevertheless, we suggest that *gphb5* could be at least an indicator of a wider process behind the LJD condition in Atlantic salmon.

In support of a possible hormonal involvement in the condition, LJD has been recently linked to dietary phosphorus (P) deficiency during early stages of development in Atlantic salmon and different dietary P physiological requirements of triploids compared to diploids (Fjelldal *et al.*, 2016). As bone in particular and the skeleton in general have a key role in endocrine regulation of minerals and nutrients (Lall and Lewis-McCrea, 2007; DiGirolamo *et al.*, 2012; Vieira *et al.*, 2013), the onset of LJD could be either the result of a pre-existing impairment in the hormonal pathways of phosphate regulation, possibly involving *gphb5* as a thyrostimulin, or that different physiological P requirements, especially in triploids, and dietary P deficiency may trigger or facilitate LJD onset with the effect displayed in the differential expression of particular genes involved in phosphate hormonal control, with a possible role for *gphb5* as a thyrostimulin. In the present study, *fgf23* was not differentially expressed between traits in both/either sample sets. This suggests that while there was no differential expression of this important P homeostasis regulator (Mangos *et al.*, 2012; Rowe, 2012; Vieira *et al.*, 2013) at the fish development stages considered, an impairment in P homeostasis may have occurred earlier, or may occur later, in the progression of LJD. Alternatively, the impairment in P

homeostasis linked to LJD in Atlantic salmon may occur through a different molecular pathway, not involving *fgf23*.

The physiological differences resulting from triploidy (e.g. fewer and bigger cells and dietary P requirements) are likely interrelated in LJD occurrence (Benfey, 2001; Fjellidal *et al.*, 2016). In addition, it has been shown in another salmonid that, although a dosage effect compensating the different cellular size between ploidies is present under normal conditions, triploidy can have deleterious effects on transcription under stress conditions (Ching *et al.*, 2009). This finding suggests that the same may occur for the occurrence and/or the exacerbation of LJD in triploid Atlantic salmon. Triploid induction in Atlantic salmon is performed by pressure shocking eggs 30 min after fertilisation, during meiosis II (Johnstone *et al.*, 1991; Benfey, 2001). The shock suppresses cell division and prevents the extrusion of a polar body, resulting in cells having three sets of chromosomes instead of two (Piferrer *et al.*, 2009). Slight variations to pressure shock timing and temperature may confer developmental variability between individuals and an uneven triploidization per egg batch. This could result in either a lower triploidy induction rate (Piferrer *et al.*, 2009) or potentially lead to imbalanced pathways where localised high levels of reactive oxygen species (ROS) occur and these then cause DNA breaks, as similar stresses (e.g. sonication) have been known to induce DNA breaks (<http://cshprotocols.cshlp.org/content/2006/4/pdb.prot4538.short>). Following the findings of the present study, *col2a1* and *gphb5* may be tested as early markers during embryonic development, in particular when Meckel's cartilage differentiation takes place, to detect potential differential expression between triploids from the same batch and as a consequence to identify possible candidates that will develop LJD in future. In a recent publication, single-nucleotide polymorphism (SNP) in *col2a1* has been associated with mandibular prognathism, a lower jaw skeletal anomaly in humans (Xue *et al.*, 2014). Similar investigation should be undertaken for the role of *col2a1* in LJD in Atlantic salmon.

In the present study, additional genes important for skeletal physiology had different regulation patterns between independent sample sets (i.e. differential expression in one but not in the other sample set). Considering that the two sample sets included fish at different developmental stages, our results suggest that fish age and/or consequent progression of the condition may influence the expression of some genes relative to others. For instance, up-regulation of both *gal* and *fn2* in LJD was detected only in the experimental sample set (the earlier developmental stage), which may be the result of a particular process occurring at that stage (due to development or condition). The process could be remodelling of the LJD affected jaw as a consequence of incorrect growth, likely driven by impaired Meckel's cartilage development, or containment of the compromised structural integrity. In fact, members of the family of both galectins and fibrillins have been shown to be involved in cartilage and bone formation and development (Barondes *et al.*, 1994; Robinson and Godfrey, 2000; Stock *et al.*, 2003; Liu and Rabinovich, 2005; Ramirez and Rifkin, 2009; Arteaga-Solis *et al.*, 2011; Bhat *et al.*, 2011; Vinik *et al.*, 2015). In particular, members of the galectins have been linked to diseases heavily affecting cartilage (i.e. rheumatoid arthritis and osteoarthritis) (Ohshima *et al.*, 2003; Guévremont *et al.*, 2004; Li *et al.*, 2008) and have a role in osteoblast differentiation, bone remodelling and osteoclastogenesis (Li *et al.*, 2008; Tanikawa *et al.*, 2010; Vinik *et al.*, 2015). On the other hand, fibrillins play a crucial role in maintaining the integrity of connective tissues, correct formation and remodelling of extracellular matrix and bone structural development with effects on morphology and mechanical properties (Ramirez and Rifkin, 2009; Arteaga-Solis *et al.*, 2011).

In the industrial sample set, the LJD fish showed down-regulation of genes important for skeletal physiology, which may support the hypothesis of the progression of the LJD condition proposed above. Our results support a scenario where cartilage impairment leads to bone development impairment that becomes more evident at later stages. For instance, down-

regulation of *igf1* in LJD may indicate impaired growth/development of cartilage and bone as well as poor mineralisation. *Igf1* is known to modulate skeletal development and in particular bone growth, resorption and mineralisation controlling proliferation and differentiation of chondrocytes, osteoblasts, osteocytes and osteoclasts (Le Roith *et al.*, 2001; Yakar *et al.*, 2002; Guntur and Rosen, 2013). Furthermore, given the possible interdependency of *igf1* and parathyroid hormone (PTH) for skeletal development (Daniel and Yongmei, 2012; Guntur and Rosen, 2013; Tahimic *et al.*, 2013) it remains unclear whether or not *igf1* down-regulation later in development and consistent down-regulation of *gphb5* in LJD in the present study may be part of the same cascade of impairment in bone hormonal control.

Bmp4 down-regulation in LJD supports our hypothesis concerning the influence of developmental stage and/or condition progression on gene expression proposed above. Bone morphogenetic proteins (BMPs), which are multi-functional growth factors of the transforming growth factor β (TGF β) superfamily, play a fundamental role for cartilage and bone development and their normal functioning is required to avoid skeletal defects or malformations (Chen *et al.*, 2004; Wang *et al.*, 2014). In particular, *bmp4* has been repeatedly associated with mandibular or maxillofacial development and shaping in fish, birds and mammals (Abzhanov *et al.*, 2004; Liu *et al.*, 2005; Hu *et al.*, 2008; Parsons and Albertson, 2009), and has been linked before to oral malformations in birds (Hu *et al.*, 2008; Bai *et al.*, 2014) and mammals (Suazo *et al.*, 2011), supporting and highlighting the possible significance of this gene in the LJD condition.

Finally, the remaining genes down-regulated in LJD-affected fish, *alp*, *colla1* and *mmp13*, probably indicate that the bone is not growing/developing correctly as a consequence of cartilaginous impairment. In fact, all of these are well-known and important skeletal structural genes: *colla1* encodes type I collagen, the main component of bone. *Alp* and *mmp13* are responsible for bone formation and mineralisation and cartilage and bone resorption,

respectively (Whyte, 1994; Karsenty and Park, 1995; Pendas *et al.*, 1997; Rossert *et al.*, 2000; Inada *et al.*, 2004; Golub and Boesze-Battaglia, 2007; Tang *et al.*, 2012). In Atlantic salmon, all these genes have been already shown to be differentially expressed in poorly mineralised and deformed vertebrae relative to normal vertebrae (Ytteborg *et al.*, 2010a; Fjelldal *et al.*, 2012b; Fjelldal *et al.*, 2016). The current study suggests their involvement in the development of a skeletal anomaly affecting the lower jaw in Atlantic salmon.

4.6 Conclusions

In conclusion, although the causes of LJD are still unknown, we have made the first contribution to the understanding of the molecular mechanisms underlying the condition. We propose *col2a1* and *gphb5* as reliable candidates for detection of the condition due to their consistent down-regulation in LJD in two independent sample sets from two developmental stages. The down-regulation of *col2a1* here may indicate that LJD in Atlantic salmon is attributable to impaired development and structural defects of Meckel's cartilage. In addition, we suggest that down-regulation and localization of *gphb5* infers a possible hormonal involvement in LJD. Although further investigation of the role of this hormone in LJD is needed, we have enhanced our understanding of a relatively novel hormone and showed for the first time that *gphb5* may be part of a mechanism behind a skeletal anomaly. Differential expression of other genes important for skeletal physiology in either one or the other sample set suggests that developmental stage or progression of the LJD condition could influence their transcription. Further molecular investigation of the marker candidates proposed in the current study is warranted. In addition, P homeostasis, skeletal hormonal control and mineralisation/structural characterisation of LJD in triploid Atlantic salmon require elucidation.

4.7 Acknowledgements

This research was supported by the Commonwealth Government's Collaborative Research Network (CRN) Program funding and the Institute for Marine and Antarctic Studies (IMAS). The authors would like to thank Petuna, Shaun Slevac and Ryan Wilkinson for their support. Ylenia Pennacchi and Deborah Leonard are thanked for technical assistance during experiments and samplings. Stephen Battaglione is thanked for his contribution to the development of this research. GA was supported by a University of Tasmania and IMAS "Tasmania Graduate Research Scholarship" and JC was supported by a University of the Sunshine Coast CRN Research Fellowship.

CHAPTER 5: GENERAL DISCUSSION

5.1 General discussion

This project has been undertaken almost 15 years after a previous major assessment of skeletal anomalies in triploid Atlantic salmon populations in Tasmania performed during another PhD project (Sadler, 2000). It also reflects a resurgence in interest around the world in the potential for farming triploid Atlantic salmon as well as more local interest in managing Tasmanian production. In fact, during the last few years, in the two main producing countries in the Northern hemisphere, Norway and Scotland, several studies have been carried out on triploid populations. These studies investigated several aspects of triploid Atlantic salmon and their general suitability for commercial production due to their appealing commercial characteristics (see Chapter 1; review by Benfey 2015). Triploids are currently and necessarily used exclusively in Tasmania to allow continuity of production and avoid early sexual maturation due to higher seawater temperatures (Benfey, 2015b). The well-established practice of commercial use of triploids in the Tasmanian Atlantic salmon industry may have also encouraged other countries to revisit earlier attempts (e.g. Johnstone *et al.*, 1991) and recently (May 2016) in Norway the production of the first triploid populations was approved (<http://www.reuters.com/article/idUSO9N17G00Y>). Nevertheless, although triploids perform equally to diploids, there are still concerns regarding the tendency of triploids to develop a higher prevalence of skeletal anomalies in particular affecting the lower jaw and the vertebral column (Benfey, 2015b). This major issue has been restraining the utilisation of triploids motivating investigation of the reasons behind the occurrence of skeletal anomalies. As a consequence, the industry has focused on trying to mitigate or solve the problem of skeletal anomalies in order to improve triploid (and diploid) welfare and production outputs.

This PhD thesis was developed in this specific context and aimed at improving our knowledge on skeletal anomalies affecting triploids (and diploids), in particular lower jaw deformity (LJD), and at producing useful information for Atlantic salmon industry, in

Tasmania and worldwide, to take into consideration during triploid production and husbandry practices. The main findings from my research are: 1) triploids are confirmed to be more prone to developing lower jaw anomalies, in particular LJD, relative to diploids, 2) LJD can occur any time in development in freshwater but there is a specific developmental stage (8-10 g) at which it starts to be first manifested, 3) elevated water temperature does not affect the prevalence of lower jaw anomalies in either ploidy in freshwater, 4) elevated water temperature affects vertebral development in both ploidies and increases occurrence of vertebral deformities more in triploids than diploids in freshwater, 5) LJD in triploids can concur with other jaw anomalies but seems to have an independent process of onset, 6) high growth rate may impact lower jaw development and facilitate occurrence, concurrence and worsening of lower jaw anomalies, 7) a cartilaginous impairment may underlie the development of LJD, 8) there is likely a hormonal involvement in LJD development, 9) condition progress and/or stage of development may influence gene expression associated with LJD, 10) the genes detected to be associated with LJD can be tested as molecular markers for the condition.

This chapter serves as a general summary of the main findings in light of the predetermined aims of this research. The findings are then discussed from a broader perspective in order to identify and suggest possible improvements for future research.

5.1.1 LJD development clues, the effect of ploidy and temperature on skeletal anomaly occurrence and considerations on opercular shortening

To fulfil the aims stated in the General Introduction, my research started with a longitudinal investigation of skeletal anomalies covering almost all the freshwater phase (Chapter 2). It was considered of critical importance to maintain diploid and triploid individuals under a meticulously controlled environment and to replicate as closely as possible industrial rearing conditions. Interestingly, the freshwater phase of Atlantic salmon is less investigated than the seawater phase in regards to skeletal anomalies occurrence, thus requires deeper analysis. This

is surprising considering that in Atlantic salmon the main morphological and developmental changes, such as skeletal mineralisation, shift from one developmental stage to another (i.e. alevin→ fry→ parr→ smolt) and smoltification, occur during the juvenile phase, in freshwater. Furthermore, in the current research, the application of an elevated temperature regime during freshwater rearing in the pre-smoltification stage to detect possible effects on skeletal anomalies prevalence in triploid (and diploid) Atlantic salmon, represents the first such attempt ever undertaken. In fact, in the hatchery the use of freshwater without temperature control, can expose fish to seasonal spikes possibly lasting for a sufficient period to affect both growth and consequent cartilage and bone physiology and leading to the occurrence of skeletal anomalies.

In the current research, the presence of LJD predominantly in triploids (Chapter 2) reinforced the hypothesis that the mechanisms underlying the occurrence may be related to the different physiology triploids do have compared to diploids. My research indicated that, although it can occur at any time in development during the freshwater phase, the onset of LJD may be in a specific period, approximately at 8-10 g and to my knowledge not earlier, in accordance with that suggested in two studies (Sadler *et al.*, 2001; Fjellidal *et al.*, 2016). This result implies that a developmental time window could be present at that stage and the lower jaw may undergo particular physiological processes creating the conditions for the first onset of LJD. The processes, possibly affecting skeletal structural morphology, could be jaw re-shaping driven by change in growth rate and/or the effect of the shift between developmental stages (i.e. from fry to parr). Further investigation of that particular stage of development is required as well as of the embryonal stage and in particular when triploid induction is delivered. In fact, the triploid induction may interfere with one or more cardinal biological processes (e.g. cell fate specification) and the deleterious consequences be manifested later. Nevertheless, the occurrence of LJD also at later stages suggests that favourable conditions for the onset can be present at any time with individual variability. Therefore, predisposition may be the common

thread in the development of LJD and the onset may be triggered or stimulated by external factors (e.g. accelerated growth and dietary deficiencies).

In the current research, a possible developmental link in triploids between LJD and shortened lower jaw (SJ), another lower jaw anomaly commonly affecting Tasmanian Atlantic salmon, was also hypothesised (Chapter 2). The hypothesis was made after observing over time a pattern in triploids of increase in LJD prevalence matching with a decrease in SJ prevalence. My initial explanation was that SJ in some individuals could have developed into a LJD at later stages. Nevertheless, following later findings (Chapter 3, discussed below) it was found that the two anomalies could be displayed concurrently. This result implied that, in the experiment described in Chapter 2, the concurrence was not detected although present. This was probably due to the difficulty in discriminating the two anomalies when concurrent and severity of SJ is just mild.

An interesting derived result from the research described in Chapter 2, concerned an unusually high prevalence (80-90%) of opercular shortening observed in both ploidies. Prevalence of opercular shortening has been frequently reported in literature but never, except for few cases, higher than 20% and its occurrence has been linked to environmental conditions and feeding behaviour (see Chapter 2). Considering that aggressive behaviour is likely to increase when fish are stocked at high densities as they have to compete more for food (Ellis *et al.*, 2002; Martins *et al.*, 2011), the use of plastic crates as replicate units to maintain fish from hatch to approximately 3 g was suggested as a possible culprit by the reviewers managing the publication of the research described in Chapter 2. In fact, at that stage fish were exposed to a higher stocking density than that recommended for Atlantic salmon at that developmental stage (50 kg m⁻³ vs. 20–30 kg m⁻³ for fish up to 30 g) (RSPCA, 2015). Nevertheless, other conditions possibly facilitating the occurrence of opercular shortening cannot be excluded (e.g. water flow and resultant fish and feed distribution and other biotic and/or abiotic factors). This

finding may be of great importance for industry and caution should be exercised when recommended stocking density cannot be applied as variable prevalence of opercular shortening may occur. This anomaly may expose affected individuals to diseases and impact their biological functions, and as a consequence performance, and product quality and value (Beraldo and Canavese, 2011; Boglione *et al.*, 2013b; Bruno *et al.*, 2013). Nevertheless, considering the reversibility of the anomalous process (recovery) (see Chapter 3), a way to mitigate opercular shortening may be available even at later stages. To better understand the phenomenon, the actual relationship between high stocking density and occurrence of opercular shortening needs to be validated. Further investigations of regeneration capabilities of the opercular tissues in Atlantic salmon should be undertaken since they have never been investigated previously.

In regards to opercular shortening, my research has also highlighted the importance of the environmental conditions as well as a possible behavioural effect on prevalence of the side affected (Chapter 2). As suggested in Chapter 2, water flow, tank design and dissimilarities in feeding behaviour between ploidies may have led to the exposure of one side rather than the other. In support of these assumptions, it was shown that triploids have different feeding behaviour and food consumption relative to diploids (Carter *et al.*, 1994) and triploidy alters brain and sensory organ morphology in Atlantic salmon (Fraser *et al.*, 2012b) possibly impacting on feeding behaviour and aggressiveness. The further impact of temperature and experimental environment on these differences, especially feeding behaviour, requires elucidation.

The current research has reported for the first time results on vertebral deformities occurrence in diploid and triploid Tasmanian populations (Chapter 2). These results allowed comparison with previous studies carried out in other countries and to confirm that also Tasmanian triploid populations tend to have a lower number of vertebrae, more deformities

and more regions of the vertebral column affected. This implies that triploids do have predisposition to those anomalous traits irrespective of the genetic background and the different rearing conditions and that, again, physiological differences linked to triploidy are likely the culprit. My research has also shown that, irrespective of the ploidy, fish exposed to an elevated temperature in freshwater have more regions of the vertebral column affected by deformities implying that inadequate temperature regimes and consequent higher growth rates may be one of the causative factors. Although no effect of temperature on prevalence of lower jaw anomalies was found, at least at the timeframe analysed, in light of the findings on vertebral deformities, the current research has pointed out that rearing temperature during freshwater phase is critical for correct vertebral development in agreement with recent findings, which showed impact of unsuitable incubation temperature on vertebral column development in Atlantic salmon (Fraser *et al.*, 2014a; Fraser *et al.*, 2015).

5.1.2 Concurrence and worsening of lower jaw anomalies, the effect of growth rate, opercular shortening recovery and deleterious effect of lower jaw anomalies

My research has demonstrated that LJD and SJ can concur in freshwater in fish either starting with a normal lower jaw or affected by SJ, both anomalies can occur at different time in development during freshwater phase and shown for the first time that both can clearly worsen over time (Chapter 3). Nevertheless, the developmental patterns observed in the two groups for both anomalies and their occurrence taking place also independently suggest that SJ and LJD have an independent onset and most likely different causative factors. If for LJD the causes seem to be mostly genetic and nutritional (Fjelldal *et al.*, 2016; Smedley *et al.*, 2016), for SJ they are unknown. Further investigations are required to understand possible causative factors for SJ. However, my research provided very useful insights into two poorly understood lower jaw skeletal anomalies that are rarely investigated in Atlantic salmon.

The high growth rate demonstrated by the fish in the experiment described in Chapter 3, compared to previous experiments reported in the literature, probably contributed to the onset and concurrence of LJD and SJ. In fact, high growth rates have been previously linked to development of skeletal anomalies (Fjelldal *et al.*, 2012c; Taylor *et al.*, 2014). This implies that fish which grow too fast may be subjected to an imbalance between body growth and skeletal development and mineralisation rate leading to bone defects. High growth rate may also underlie the worsening of LJD and the development of SJ. In the case of SJ, my research suggested that the lower jaw failed to grow at the same rate as the upper jaw and thus remained shorter. In addition, the observation of fish recovering from SJ may imply the possible restoration of the differential development between upper and lower jaw. This finding reinforces the theory that growth rate could influence that morphological imbalance. Interrelation between growth rate and supplementation of fundamental dietary elements as well as possible genetic predisposition for SJ, as well as LJD, require elucidation. The use of tagged individuals would have improved aspects of the current research and helped to better understand the processes. Unfortunately, it was not possible to perform it due to technical issues such as difficulties in tagging individuals at the starting size and durability of the type of tag chosen. Tagging would have allowed to detect accurate onset time and to track the development of lower jaw skeletal anomalies as well as morphological changes (i.e. worsening or recovery) in the lower jaw over time in individual fish. Nevertheless, the typology of both LJD and SJ conditions does not allow to perform repeated molecular analysis on the tissue of the same individual over time which must be sacrificed not permitting a full understanding of the processes occurring at intermediate stages of the trait development. In addition, while morphological and histological analyses of LJD have been carried out before (Hughes, 1992), none is available for SJ. Further detailed histological analyses may improve our understanding and better characterise the two conditions.

My research confirmed the previously reported anecdotal observations on the deleterious effect of lower jaw anomalies on specific growth rate (SGR) (Chapter 3). It is likely that an impairment of the lower jaw functioning may limit the efficiency in feeding. In addition, impact on respiratory abilities could also contribute to the exacerbation of the issue (Lijalad and Powell, 2009). Feed intake measure and individual tagging would help to confirm this finding. Nevertheless, in support of that, just recently fish affected by lower jaw skeletal anomalies have been found to weigh 20% less than normal fish at harvest (Smedley *et al.*, 2016). This result confirms that over time fish with an impaired jaw grow slower relative to normal fish.

The findings described in Chapter 3 have also contributed to the understanding of another phenomenon poorly analysed in the literature. To my knowledge, the current research showed for the first time that Atlantic salmon recover from opercular shortening with a process of re-growth of the tissue. Recovery from opercular shortening in diploid Atlantic salmon was observed before but the results were only reported in a PhD thesis and not published (MacLean, 1999). The fact that recovery from opercular shortening was slower in individuals affected by lower jaw skeletal anomalies strongly supports the aforementioned theory concerning their detrimental effects on fish feeding and consequent performance as well as the possible interconnection with respiratory impairment. In that case, respiratory impairment would be a consequence of both opercular shortening and lower jaw anomaly likely impacting even more on fish performance.

The developmental patterns observed for the prevalence of misaligned jaw (MA), a lower jaw anomaly rarely reported in the literature, showed that MA is more likely to occur in an already deformed lower jaw (i.e. LJD or SD) (Chapter 3). This finding implies that an impairment in the structural integrity of the lower jaw may lead to the development of more severe anomalies as well as expose it to other invalidating factors (e.g. injuries). There is still

uncertainty on whether or not MA is just the result of compromised structural integrity or environmental factors (i.e. physical injury, as reported for other fish species). Nevertheless, MA prevalence is usually very low, with negligible production impact.

5.1.3 Considerations on lower jaw plasticity

Overall, combining the findings of Chapter 2 and 3, my research demonstrated that during freshwater phase and at the developmental time frame analysed, the lower jaw in Atlantic salmon is a very plastic anatomical structure that might be subjected to dramatic structural changes in a relatively short time (< one month). This consideration is in light of the changes in prevalence, concurrence and worsening of the lower jaw skeletal anomaly categories observed during the assessments performed. These findings have pointed out the likely effect of growth rate (and adequate dietary elements supply?) on a fast changing tissue like the lower jaw in Atlantic salmon, and the evident susceptibility of triploids to lower jaw anomalies.

5.1.4 Triploids and dietary requirements

It has been recently shown that triploids may have different dietary phosphorus (P) requirements compared to diploids and that occurrence of LJD, as well as of vertebral deformities, might be linked to inadequate supply of dietary P during juvenile phase in triploids in both freshwater and seawater (Fjelldal *et al.*, 2016; Smedley *et al.*, 2016). Nevertheless, the relationship between LJD and P deficiency needs to be further investigated especially in light of the current research where adequate supply of dietary P did not prevent the occurrence of LJD (Chapter 2). The possible effect of different requirements of other dietary elements in triploids cannot be excluded as well as the effect of the diet specific formulation. Similar experiments to the ones mentioned above are recommended for Tasmanian triploid (and diploid) populations to verify that dietary requirements are the main factor to improve in order to mitigate the occurrence of skeletal anomalies and to exclude genetic background/predisposition of Tasmanian populations as additional causative factor.

In light of the above, there may be the need to design specific diets for triploids to meet their different dietary requirements or at least to improve the diets used for diploid populations to make them suitable for triploids. Furthermore, the current research suggests that both diploid and triploid fish reared at higher temperature in freshwater might require even more specific diet formulations. The same may be required in seawater as well. New diets should be formulated taking into account that fish, not necessarily only triploids, may experience higher growth rates and higher nutrient requirements that drive changes in feeding behaviour as a result of higher temperature (Miller *et al.*, 2006; Carter *et al.*, 2010). Suitable diets for fish reared at higher temperatures would help fish, in particular triploids, to cope with possible imbalances between body growth and bone mineralisation (Fjelldal *et al.*, 2016; Smedley *et al.*, 2016).

Furthermore, climate change and global warming are driving a global increase in average water temperature and its effects may heavily impact on Atlantic salmon production, especially in Tasmania. In fact, as mentioned above, in Tasmania higher water temperatures are present compared to the Northern hemisphere and it is not known whether or not a further increase may compromise Atlantic salmon farming. In light of the deleterious effect of elevated water temperature on fish skeletal development, in both diploids and triploids, countermeasures need to be adopted not to jeopardize fish welfare and to guarantee correct growth and utilisation of dietary elements.

5.1.5 Molecular characterisation of LJD and detection of possible markers

My research reported for the first time results concerning the molecular mechanisms underlying LJD condition (Chapter 4). The aim was to characterise LJD at the molecular level. The use of different molecular techniques has allowed to detect important candidate genes involved in the process and to suggest mechanisms as well as the tissue responsible for this skeletal anomaly affecting the lower jaw of triploid Atlantic salmon. Two genes in particular,

col2a1 and *gphb5*, showed an abnormal and consistent expression pattern between the two independent sample sets used. The downregulation of both genes in the lower jaw tissue of LJD-affected individuals highlighted their likely role in the development of the condition. On one hand, differential expression of *col2a1*, a specific marker of cartilage, has suggested which type of tissue might be responsible for the trait, on the other hand the differential expression of *gphb5*, a recently discovered hormone also known as thyrostimulin, has indicated a possible hormonal role in the condition.

Since Meckel's cartilage is the main cartilaginous tissue in the lower jaw, supporting the dentary bone, my research has suggested that downregulation of *col2a1* indicates an impairment in this tissue. An anomalous development of such important tissue for lower jaw structural integrity can definitely impact on the consequent development of the supported bone leading to an incorrect growth trajectory. As observed in histological analysis performed in the current research (data not published) and as reported by Hughes (1992) Meckel's cartilage definitely appears bent and deformed (and consequently the dentary bone is too) in individuals affected with LJD. Nevertheless, further analysis should be undertaken to verify the condition of specific cartilage and bone cells in the tissue/s affected.

The result concerning *gphb5* might be of interest to a broader community of biologists. In fact, the real role of *gphb5* is still poorly understood although recently it has been linked to skeletal physiology supporting, to some extent, what the current research proposes (Bassett *et al.*, 2015). To my knowledge, the current research showed for the first time that *gphb5* may be involved in the development of a skeletal anomaly in fish, implying a possible similar scenario in other vertebrates. As *gphb5* is likely associated with thyroid organ function (Nakabayashi *et al.*, 2002) and since thyroid hormones are well known to regulate skeletal development (Bassett *et al.*, 2007; Bassett *et al.*, 2015), it would be worthwhile to investigate possible differences in thyroid morphology/functioning between normal and LJD-affected individuals.

Finally, the findings of my research allowed to make a hypothesis about the effect of stage of development and/or condition progression on the regulation of other important genes for skeletal physiology. In fact, most genes tested were found differentially expressed in one sample set but not in the other, with the exception of *col2a1* and *gphb5*. While the functional significance of the latter two factors in LJD manifestation is poorly understood, the current research proposes to further investigate them which could be possibly used as markers for LJD development. Further research may lead to a better characterisation of LJD. In particular, higher depth and broader molecular investigations (e.g. Single Nucleotide Polymorphism array or RAD-Seq) might reveal mutations or variations in the sequence of genes fundamental for skeletal development (included the ones proposed in this research). A similar assessment has been recently performed for jaw deformity in another species (yellowtail kingfish) by Patel *et al.* (2016). Considering the link between triploidy and LJD, the triploidy induction event may be likely responsible for the occurrence of mutations in part of the population subjected to the shock. This may lead those individuals to predisposition to the condition that can manifest at different times and/or if they are exposed to inadequate husbandry conditions. Nevertheless, other options than just genetic imbalance (i.e. the aforementioned deleterious effect of triploidy on transcription) cannot be excluded. In fact, there could be other unknown impairments in critical developmental processes which only cross-comparison with other model species where the mechanism is known might help to detect.

REFERENCES

- Abzhanov, A., Protas, M., Grant, B.R., Grant, P.R., Tabin, C.J., 2004. Bmp4 and morphological variation of beaks in Darwin's finches. *Science* 305, 1462-1465.
- Allendorf, F.W., Leary, R.F., 1984. Heterozygosity in gynogenetic diploids and triploids estimated by gene-centromere recombination rates. *Aquaculture* 43, 413-420.
- Amoroso, G., Adams, M.B., Ventura, T., Carter, C.G., Cobcroft, J.M., 2016a. Skeletal anomaly assessment in diploid and triploid juvenile Atlantic salmon (*Salmo salar* L.) and the effect of temperature in freshwater. *Journal of Fish Diseases* 39, 449-466.
- Amoroso, G., Cobcroft, J.M., Adams, M.B., Ventura, T., Carter, C.G., 2016b. Concurrence of lower jaw skeletal anomalies in triploid Atlantic salmon (*Salmo salar* L.) and the effect on growth in freshwater. *Journal of Fish Diseases* DOI: 10.1111/jfd.12492.
- Apschner, A., Schulte-Merker, S., Witten, P.E., 2011. Chapter 10 - Not All Bones are Created Equal – Using Zebrafish and Other Teleost Species in Osteogenesis Research. in: H. William Detrich, M.W., Leonard, I.Z. (Eds.), *Methods in Cell Biology*. Academic Press, pp. 239-255.
- Arteaga-Solis, E., Sui-Arteaga, L., Kim, M., Schaffler, M.B., Jepsen, K.J., Pleshko, N., Ramirez, F., 2011. Material and mechanical properties of bones deficient for fibrillin-1 or fibrillin-2 microfibrils. *Matrix Biology* 30, 188-194.
- Asgard, T., Holmefjord, I., Gjerde, B., Baeverfjord, G., 1996. Ryggdeformitet hos laks. AKVAFORSK-rapport 6/96, 18 pp.
- Atkins, M.E., Benfey, T.J., 2008. Effect of acclimation temperature on routine metabolic rate in triploid salmonids. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology* 149, 157-161.
- Aunsmo, A., Guttvik, A., Midtlyng, P.J., Larssen, R.B., Evensen, O., Skjerve, E., 2008. Association of spinal deformity and vaccine-induced abdominal lesions in harvest-sized Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases* 31, 515-524.
- Baeverfjord, G., Asgard, T., Shearer, K.D., 1998. Development and detection of phosphorus deficiency in Atlantic salmon, *Salmo salar* L., parr and post-smolts. *Aquaculture Nutrition* 4, 1-11.
- Bai, H., Zhu, J., Sun, Y., Liu, R., Liu, N., Li, D., Wen, J., Chen, J., 2014. Identification of genes related to beak deformity of chickens using digital gene expression profiling. *PLoS ONE* 9, e107050.
- Barondes, S.H., Cooper, D.N., Gitt, M.A., Leffler, H., 1994. Galectins. Structure and function of a large family of animal lectins. *Journal of Biological Chemistry* 269, 20807-20810.
- Bassett, J.H.D., Nordström, K., Boyde, A., Howell, P.G.T., Kelly, S., Vennström, B., Williams, G.R., 2007. Thyroid Status during Skeletal Development Determines Adult Bone Structure and Mineralization. *Molecular Endocrinology* 21, 1893-1904.
- Bassett, J.H.D., van der Spek, A., Logan, J.G., Gogakos, A., Bagchi-Chakraborty, J., Murphy, E., van Zeijl, C., Down, J., Croucher, P.I., Boyde, A., Boelen, A., Williams, G.R., 2015. Thyrostimulin regulates osteoblastic bone formation during early skeletal development. *Endocrinology* 156, 3098-3113.
- Battaglene, S., Carter, C., Hobday, A.J., Lyne, V., Nowak, B., 2008. Scoping Study into Adaptation of the Tasmanian Salmonid Aquaculture Industry to Potential Impacts of Climate Change. National Agriculture & Climate Change Action Plan: Implementation Programme report 84p.
- Benfey, T.J., 1999. The physiology and behavior of triploid fishes. *Reviews in Fisheries Science* 7, 39-67.
- Benfey, T.J., 2001. Use of sterile triploid Atlantic salmon (*Salmo salar* L.) for aquaculture in New Brunswick, Canada. *Ices Journal of Marine Science* 58, 525-529.

- Benfey, T.J., 2009. Producing sterile and single-sex populations of fish for aquaculture. In: Burnell, G., Allan, G. (Eds.), *New Technologies in Aquaculture: Improving Production Efficiency, Quality and Environmental Management*. Woodhead Publishing Ltd., Cambridge, United Kingdom, pp. 143-164.
- Benfey, T.J., 2015a. Biocontainment measures to reduce/mitigate potential post-escape interactions between cultured European-origin and wild native Atlantic salmon in Newfoundland. DFO Can. Sci. Advis. Sec. Res. Doc. 2015/003. v + 28 p.
- Benfey, T.J., 2015b. Effectiveness of triploidy as a management tool for reproductive containment of farmed fish: Atlantic salmon (*Salmo salar*) as a case study. *Reviews in Aquaculture*, n/a-n/a.
- Benfey, T.J., Sutterlin, A.M., 1984a. Growth and gonadal development in triploid landlocked Atlantic salmon (*Salmo salar*). *Can. J. Fish. Aquat. Sci.* 41, 1387-1392.
- Benfey, T.J., Sutterlin, A.M., 1984b. Triploidy induced by heat shock and hydrostatic pressure in landlocked Atlantic salmon (*Salmo salar* L.). *Aquaculture* 36, 359-367.
- Benfey, T.J., Sutterlin, A.M., Thompson, R.J., 1984. Use of erythrocyte measurements to identify triploid Salmonids. *Canadian Journal of Fisheries and Aquatic Sciences* 41, 980-984.
- Beraldo, P., Canavese, B., 2011. Recovery of opercular anomalies in gilthead sea bream, *Sparus aurata* L.: morphological and morphometric analysis. *Journal of Fish Diseases* 34, 21-30.
- Berg, A., Yurtseva, A., Hansen, T., Lajus, D., Fjelldal, P.G., 2012. Vaccinated farmed Atlantic salmon are susceptible to spinal and skull deformities. *Journal of Applied Ichthyology* 28, 446-452.
- Berg, A.E., Rødseth, O.M., Tangeras, A., Hansen, T.J., 2006. Time of vaccination influences development of adherences, growth and spinal deformities in Atlantic salmon (*Salmo salar* L.). *Dis. Aquat. Org.* 69, 239-248.
- Bhat, R., Lerea, K., Peng, H., Kaltner, H., Gabius, H.-J., Newman, S., 2011. A regulatory network of two galectins mediates the earliest steps of avian limb skeletal morphogenesis. *Bmc Developmental Biology* 11, 6.
- Bodó, E., Kany, B., Gáspár, E., Knüver, J., Kromminga, A., Ramot, Y., Bíró, T., Tiede, S., van Beek, N., Poeggeler, B., Meyer, K.C., Wenzel, B.E., Paus, R., 2010. Thyroid-Stimulating Hormone, a novel, locally produced modulator of human epidermal functions, is regulated by Thyrotropin-Releasing Hormone and Thyroid Hormones. *Endocrinology* 151, 1633-1642.
- Boglione, C., Gavaia, P., Koumoundouros, G., Gisbert, E., Moren, M., Fontagné, S., Witten, P.E., 2013a. Skeletal anomalies in reared European fish larvae and juveniles. Part 1: normal and anomalous skeletogenic processes. *Reviews in Aquaculture* 5, S99-S120.
- Boglione, C., Gisbert, E., Gavaia, P., Witten, P.E., Moren, M., Fontagné, S., Koumoundouros, G., 2013b. Skeletal anomalies in reared European fish larvae and juveniles. Part 2: main typologies, occurrences and causative factors. *Reviews in Aquaculture* 5, S121-S167.
- Boyle, W.J., Simonet, W.S., Lacey, D.L., 2003. Osteoclast differentiation and activation. *Nature* 423, 337-342.
- Branson, E.J., Nieto, D., 1999. Jaw deformities in Atlantic salmon (*Salmo salar*) in Chile. *Proceedings of the 9th International European Association of Fish Pathology Conference, Rhodes, Greece*. p. 31 ABSTRACT.
- Bruno, D.W., 1990. Jaw deformity associated with farmed Atlantic salmon (*Salmo salar*). *Veterinary Record* 126, 402-403.
- Bruno, D.W., Noguera, P.A., Poppe, T.T., 2013. *A Colour Atlas of Salmonid Diseases*, 2nd edn. Springer, Dordrecht, Heidelberg, New York, London. pp. 220.

- Burnley, T.A., Stryhn, H., Burnley, H.J., Hammell, K.L., 2010. Randomized clinical field trial of a bacterial kidney disease vaccine in Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases* 33, 545-557.
- Carter, C.G., Katersky, R.S., Barnes, J., Hauler, R.C., Bridle, A.R., 2010. Redefining nutrient requirements of fish in sub-optimum environments. In: *Energy and Protein Metabolism and Nutrition*. Edited by Crovetto, G.M., EAAP Publication 127, 445-446.
- Carter, C.G., McCarthy, I.D., Houlihan, D.F., Johnstone, R., Walsingham, M.V., Mitchell, A.I., 1994. Food consumption, feeding behaviour and growth of diploid and triploid Atlantic salmon, *Salmo salar* L., parr. *Canadian Journal of Zoology* 72, 609-617.
- Cheah, K.S., Stoker, N.G., Griffin, J.R., Grosveld, F.G., Solomon, E., 1985. Identification and characterization of the human type II collagen gene (COL2A1). *Proc Natl Acad Sci USA* 82(9), 2555-2559.
- Cheah, K.S., Au, P.K., Lau, E.T., Little, P.F., Stubbs, L., 1991. The mouse COL2A1 gene is highly conserved and linked to int-1 on chromosome 15. *Mamm Genome* 1, 171-183.
- Chen, D., Zhao, M., Mundy, G.R., 2004. Bone Morphogenetic Proteins. *Growth Factors* 22, 233-241.
- Ching, B., Jamieson, S., Heath, J.W., Heath, D.D., Hubberstey, A., 2009. Transcriptional differences between triploid and diploid Chinook salmon (*Oncorhynchus tshawytscha*) during live *Vibrio anguillarum* challenge. *Heredity* 104, 224-234.
- Cobcroft, J.M., Battaglione, S.C., 2009. Jaw malformation in striped trumpeter *Latris lineata* larvae linked to walling behaviour and tank colour. *Aquaculture* 289, 274-282.
- Cobcroft, J.M., Battaglione, S.C., 2013. Skeletal malformations in Australian marine finfish hatcheries. *Aquaculture* 396, 51-58.
- Cobcroft, J.M., Pankhurst, P.M., Poortenaar, C., Hickman, B., Tait, M., 2004. Jaw malformation in cultured yellowtail kingfish (*Seriola lalandi*) larvae. *New Zealand Journal of Marine and Freshwater Research* 38, 67-71.
- Cotter, D., O'Donovan, V., Drumm, A., Roche, N., Ling, E.N., Wilkins, N.P., 2002. Comparison of fresh water and marine performances of all-female diploids and triploid Atlantic salmon (*Salmo salar*). *Aquac. Res.* 33, 43-53.
- Cotterell, S.P., Wardle, C.S., 2004. Endurance swimming of diploid and triploid Atlantic salmon. *J. Fish Biol.* 65, 55-68.
- Daniel, D.B., Yongmei, W., 2012. Insulin Like Growth Factor-I: A critical mediator of the skeletal response to Parathyroid Hormone. *Current Molecular Pharmacology* 5, 135-142.
- Delaissé, J.-M., Andersen, T.L., Engsig, M.T., Henriksen, K., Troen, T., Blavier, L., 2003. Matrix metalloproteinases (MMP) and cathepsin K contribute differently to osteoclastic activities. *Microscopy Research and Technique* 61, 504-513.
- DiGirolamo, D.J., Clemens, T.L., Kousteni, S., 2012. The skeleton as an endocrine organ. *Nat Rev Rheumatol* 8, 674-683.
- Donahue, L.R., Chang, B.O., Mohan, S., Miyakoshi, N., Wergedal, J.E., Baylink, D.J., Hawes, N.L., Rosen, C.J., Ward-Bailey, P., Zheng, Q.Y., Bronson, R.T., Johnson, K.R., Davisson, M.T., 2003. A Missense mutation in the mouse Col2a1 gene causes spondyloepiphyseal dysplasia congenita, hearing loss, and retinoschisis. *Journal of Bone and Mineral Research* 18, 1612-1621.
- Eddy, F.B., Williams, E.M., 1987. Nitrite and Freshwater Fish. *Chemistry and Ecology* 3, 1-38.
- Ellis, T., North, B., Scott, A.P., Bromage, N.R., Porter, M., Gadd, D., 2002. The relationships between stocking density and welfare in farmed rainbow trout. *Journal of Fish Biology* 61, 493-531.

- Erlebacher, A., Filvaroff, E.H., Gitelman, S.E., Derynck, R., 1995. Toward a molecular understanding of skeletal development. *Cell* 80, 371-378.
- FAO, 2016a. FAO 2004-2016. Cultured Aquatic Species Information Programme. *Salmo salar*. Cultured Aquatic Species Information Programme. Text by Jones, M. In: FAO Fisheries and Aquaculture Department [online]. Rome. Updated 1 January 2004. [Cited 12 January 2016]. http://www.fao.org/fishery/culturedspecies/Salmo_salar/en.
- FAO, 2016b. FAO - Fisheries and Aquaculture Information and Statistics Branch. <http://www.fao.org/fishery/statistics/global-aquaculture-production/query/en>.
- Fernández, I., Hontoria, F., Ortiz-Delgado, J.B., Kotzamanis, Y., Estévez, A., Zambonino-Infante, J.L., Gisbert, E., 2008. Larval performance and skeletal deformities in farmed gilthead sea bream (*Sparus aurata*) fed with graded levels of Vitamin A enriched rotifers (*Brachionus plicatilis*). *Aquaculture* 283, 102-115.
- Fjelldal, P.G., Hansen, T., 2010. Vertebral deformities in triploid Atlantic salmon (*Salmo salar* L.) underyearling smolts. *Aquaculture* 309, 131-136.
- Fjelldal, P.G., Nordgarden, U., Hansen, T., 2007a. The mineral content affects vertebral morphology in underyearling smolt of Atlantic salmon (*Salmo salar* L.). *Aquaculture* 270, 231-239.
- Fjelldal, P.G., Hansen, T.J., Berg, A.E., 2007b. A radiological study on the development of vertebral deformities in cultured Atlantic salmon (*Salmo salar* L.). *Aquaculture* 273, 721-728.
- Fjelldal, P.G., Hansen, T., Albrektsen, S., 2012a. Inadequate phosphorus nutrition in juvenile Atlantic salmon has a negative effect on long-term bone health. *Aquaculture* 334, 117-123.
- Fjelldal, P.G., Nordgarden, U., Wargelius, A., Taranger, G.L., Waagbo, R., Olsen, R.E., 2010. Effects of vegetable feed ingredients on bone health in Atlantic salmon. *Journal of Applied Ichthyology* 26, 327-333.
- Fjelldal, P.G., Lock, E.J., Hansen, T., Wargelius, A., El-Mowafi, A., Ørnsrud, R., 2011. Extra dietary phosphorous prevents vertebral deformities in triploid Atlantic salmon (*Salmo salar* L.) smolts. *Interdisciplinary Approach in Fish Skeletal Biology Second Meeting*, meeting abstract, p. 33, 26-28 April, Tavira, Portugal
- Fjelldal, P.G., Nordgarden, U., Berg, A., Grotmol, S., Totland, G.K., Wargelius, A., Hansen, T., 2005. Vertebrae of the trunk and tail display different growth rates in response to photoperiod in Atlantic salmon, *Salmo salar* L., post-smolts. *Aquaculture* 250, 516-524.
- Fjelldal, P.G., Lock, E.-J., Grotmol, S., Totland, G.K., Nordgarden, U., Flik, G., Hansen, T., 2006. Impact of smolt production strategy on vertebral growth and mineralisation during smoltification and the early seawater phase in Atlantic salmon (*Salmo salar*, L.). *Aquaculture* 261, 715-728.
- Fjelldal, P.G., Hansen, T., Breck, O., Sandvik, R., Waagbo, R., Berg, A., Ornsrud, R., 2009. Supplementation of dietary minerals during the early seawater phase increase vertebral strength and reduce the prevalence of vertebral deformities in fast-growing under-yearling Atlantic salmon (*Salmo salar* L.) smolt. *Aquaculture Nutrition* 15, 366-378.
- Fjelldal, P.G., Lock, E.J., Hansen, T., Waagbo, R., Wargelius, A., Martens, L.G., El-Mowafi, A., Ornsrud, R., 2012b. Continuous light induces bone resorption and affects vertebral morphology in Atlantic salmon (*Salmo salar* L.) fed a phosphorous deficient diet. *Aquaculture Nutrition* 18, 610-619.
- Fjelldal, P.G., Hansen, T., Breck, O., Ornsrud, R., Lock, E.J., Waagbo, R., Wargelius, A., Witten, P.E., 2012c. Vertebral deformities in farmed Atlantic salmon (*Salmo salar* L.) - etiology and pathology. *Journal of Applied Ichthyology* 28, 433-440.
- Fjelldal, P.G., Hansen, T.J., Lock, E.J., Wargelius, A., Fraser, T.W.K., Sambraus, F., El-Mowafi, A., Albrektsen, S., Waagbø, R., Ørnsrud, R., 2016. Increased dietary

- phosphorous prevents vertebral deformities in triploid Atlantic salmon (*Salmo salar* L.). *Aquaculture Nutrition* 22, 72-90.
- Forseth, T., Hurley, M.A., Jensen, A.J., Elliott, J.M., 2001. Functional models for growth and food consumption of Atlantic salmon parr, *Salmo salar*, from a Norwegian river. *Freshwater Biology* 46, 173-186.
- Fraser, T.W.K., Fjelldal, P.G., Hansen, T., Mayer, I., 2012a. Welfare considerations of triploid fish. *Reviews in Fisheries Science* 20, 192-211.
- Fraser, T.W.K., Hansen, T., Fleming, M.S., Fjelldal, P.G., 2015. The prevalence of vertebral deformities is increased with higher egg incubation temperatures and triploidy in Atlantic salmon *Salmo salar* L. *Journal of Fish Diseases* 38, 75-89.
- Fraser, T.W.K., Fjelldal, P.G., Skjæraasen, J.E., Hansen, T., Mayer, I., 2012b. Triploidy alters brain morphology in pre-smolt Atlantic salmon *Salmo salar*: possible implications for behaviour. *Journal of Fish Biology* 81, 2199-2212.
- Fraser, T.W.K., Fleming, M.S., Poppe, T.T., Hansen, T., Fjelldal, P.G., 2014a. The effect of ploidy and incubation temperature on survival and the prevalence of aplasia of the septum transversum in Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases* 37, 189-200.
- Fraser, T.W.K., Hansen, T., Skjæraasen, J.E., Mayer, I., Sambraus, F., Fjelldal, P.G., 2013. The effect of triploidy on the culture performance, deformity prevalence, and heart morphology in Atlantic salmon. *Aquaculture* 416-417, 255-264.
- Fraser, T.W.K., Hansen, T., Mayer, I., Skjæraasen, J.E., Glover, K.A., Sambraus, F., Fjelldal, P.G., 2014b. The effect of triploidy on vaccine side-effects in Atlantic salmon. *Aquaculture* 433, 481-490.
- Galbreath, P.F., Thorgaard, G.H., 1995. Saltwater performance of all female triploid Atlantic salmon. *Aquaculture* 138, 77-85.
- Galbreath, P.F., St Jean, W., Anderson, V., Thorgaard, G.H., 1994. Freshwater performance of all-female diploid and triploid Atlantic salmon. *Aquaculture* 128, 41-49.
- Garofalo, S., Vuorio, E., Metsaranta, M., Rosati, R., Toman, D., Vaughan, J., Lozano, G., Mayne, R., Ellard, J., Horton, W., 1991. Reduced amounts of cartilage collagen fibrils and growth plate anomalies in transgenic mice harboring a glycine-to-cysteine mutation in the mouse type II procollagen alpha 1-chain gene. *Proceedings of the National Academy of Sciences* 88, 9648-9652.
- Gil-Martens, L., 2010. Inflammation as a potential risk factor for spinal deformities in farmed Atlantic salmon (*Salmo salar* L.). *J. Appl. Ichthyol.* 26, 350-354.
- Gil-Martens, L., Lock, E.J., Fjelldal, P.G., Wargelius, A., Araujo, P., Torstensen, B.E., Witten, P.E., Hansen, T., Waagbø, R., Ørnsrud, R., 2010. Dietary fatty acids and inflammation in the vertebral column of Atlantic salmon, *Salmo salar* L., smolts: a possible link to spinal deformities. *J. Fish Dis.* 33, 957-972.
- Gillis, J.A., Witten, P.E., Hall, B.K., 2006. Chondroid bone and secondary cartilage contribute to apical dentary growth in juvenile Atlantic salmon. *Journal of Fish Biology* 68, 1133-1143.
- Gjerde, B., Pante, M.J.R., Baevefjord, G., 2005. Genetic variation for a vertebral deformity in Atlantic salmon (*Salmo salar*). *Aquaculture* 244, 77-87.
- Glover, K., Madhun, A., Dahle, G., Sorvik, A., Wennevik, V., Skaala, O., Morton, H., Hansen, T., Fjelldal, P., 2015. The frequency of spontaneous triploidy in farmed Atlantic salmon produced in Norway during the period 2007-2014. *BMC Genetics* 16, 37.
- Glover, K.A., Bos, J.B., Urdal, K., Madhun, A.S., Sørvik, A.G.E., Unneland, L., Seliussen, B.B., Skaala, Ø., Skilbrei, O.T., Tang, Y., Wennevik, V., 2016. Genetic screening of farmed Atlantic salmon escapees demonstrates that triploid fish display reduced migration to freshwater. *Biological Invasions* 18, 1287-1294.

- Goicoechea, O., Enriquez, R., Paredes, E., Molinari, E., 1999. Jaw and other skeletal deformities in Chilean farmed Atlantic salmon (*Salmo salar*). Proceedings of the 9th International European Association of Fish Pathology Conference, Rhodes, Greece. p. 31 ABSTRACT.
- Golub, E.E., Boesze-Battaglia, K., 2007. The role of alkaline phosphatase in mineralization. *Current Opinion in Orthopaedics* 18.
- Grini, A., Hansen, T., Berg, A., Wargelius, A., Fjelldal, P.G., 2011. The effect of water temperature on vertebral deformities and vaccine-induced abdominal lesions in Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases* 34, 531-546.
- Gross, M.R., 1998. One species with two biologies: Atlantic salmon (*Salmo salar*) in the wild and in aquaculture. *Canadian Journal of Fisheries and Aquatic Sciences* 55, 131-144.
- Grotmol, S., Kryvi, H., Nordvik, K., Totland, G., 2003. Notochord segmentation may lay down the pathway for the development of the vertebral bodies in the Atlantic salmon. *Anatomy and Embryology* 207, 263-272.
- Grotmol, S., Nordvik, K., Kryvi, H., Totland, G.K., 2005. A segmental pattern of alkaline phosphatase activity within the notochord coincides with the initial formation of the vertebral bodies. *Journal of Anatomy* 206, 427-436.
- Guévremont, M., Martel-Pelletier, J., Boileau, C., Liu, F.T., Richard, M., Fernandes, J.C., Pelletier, J.P., Reboul, P., 2004. Galectin-3 surface expression on human adult chondrocytes: a potential substrate for collagenase-3. *Annals of the Rheumatic Diseases* 63, 636-643.
- Guntur, A.R., Rosen, C.J., 2013. IGF-1 regulation of key signaling pathways in bone. *BoneKEY Rep* 2.
- Hall, B.K., 2015. *Bones and Cartilage: Developmental and Evolutionary Skeletal Biology* 2nd Edition. Elsevier (Ed.), London. pp. 920.
- Hall, B.K., Witten, P.E., 2007. Plasticity of and transitions between skeletal tissues in vertebrate evolution and development. In: Anderson JS, Sues H-D (eds) *Major Transitions in Vertebrate Evolution*, pp. 13-56. Indiana University Press, Bloomington, IN.
- Handeland, S.O., Stefansson, S.O., 2001. Photoperiod control and influence of body size on off-season parr-smolt transformation and post-smolt growth. *Aquaculture* 192, 291-307.
- Handeland, S.O., Imsland, A.K., Stefansson, S.O., 2008. The effect of temperature and fish size on growth, feed intake, food conversion efficiency and stomach evacuation rate of Atlantic salmon post-smolts. *Aquaculture* 283, 36-42.
- Handeland, S.O., Bjornsson, B.T., Arnesen, A.M., Stefansson, S.O., 2003. Seawater adaptation and growth of post-smolt Atlantic salmon (*Salmo salar*) of wild and farmed strains. *Aquaculture* 220, 367-384.
- Handeland, S.O., Imsland, A.K., Ebbesson, L.O.E., Nilsen, T.O., Hosfeld, C.D., Baevefjord, G., Espmark, A., Rosten, T., Skilbrei, O.T., Hansen, T., Gunnarsson, G.S., Breck, O., Stefansson, S.O., 2013. Low light intensity can reduce Atlantic salmon smolt quality. *Aquaculture* 384, 19-24.
- Hansen, T., Fjelldal, P.G., Yurtseva, A., Berg, A., 2010. A possible relation between growth and number of deformed vertebrae in Atlantic salmon (*Salmo salar* L.). *Journal of Applied Ichthyology* 26, 355-359.
- Hansen, T.J., Olsen, R.E., Stien, L., Oppedal, F., Torgersen, T., Breck, O., Remen, M., Vågseth, T., Fjelldal, P.G., 2015. Effect of water oxygen level on performance of diploid and triploid Atlantic salmon post-smolts reared at high temperature. *Aquaculture* 435, 354-360.

- Hobday, A.J., Poloczanska, E.S., Matear, R.J., 2008. Implications of Climate Change for Australian Fisheries and Aquaculture: a preliminary assessment. Report to the Department of Climate Change, Canberra, Australia. August 2008. .
- Hsu, S.Y., Nakabayashi, K., Bhalla, A., 2002. Evolution of glycoprotein hormone subunit genes in bilateral Metazoa: identification of two novel human glycoprotein hormone subunit family genes, GPA2 and GPB5. *Molecular Endocrinology* 16, 1538-1551.
- Hu, D., Colnot, C., Marcucio, R.S., 2008. The effect of BMP signaling on development of the jaw skeleton. *Developmental dynamics: an official publication of the American Association of Anatomists* 237, 3727-3737.
- Hughes, D., 1992. Lower jaw deformity in farmed Tasmanian Atlantic salmon *Salmo salar* (Salmoniformes, Teleostei). Final report. Barriers and Breakthroughs. Papers from 1992 SALTAS Research and Development Seminar. SALTAS, Hobart, Tasmania, pp. 17-64.
- Inada, M., Wang, Y.M., Byrne, M.H., Rahman, M.U., Miyaura, C., Lopez-Otin, C., Krane, S.M., 2004. Critical roles for collagenase-3 (Mmp13) in development of growth and in endochondral plate cartilage ossification. *Proceedings of the National Academy of Sciences of the United States of America* 101, 17192-17197.
- Javed, A., Chen, H., Ghorri, F.Y., 2010. Genetic and transcriptional control of bone formation. *Oral Maxillofac Surg Clin North Am* 22, 283-293.
- Jobling, M., Arnesen, A.M., Benfey, T., Carter, C., Hardy, R., Francois, N.R.L., O'Keefe, R., Koskela, J., Lamarre, S.G., 2010. The salmonids (family: Salmonidae). In: Francois, N.R.L., Jobling, M., Carter, C. and Blier, P. (eds.). *Finfish Aquaculture Diversification*. CAB International, Wallingford, UK, chapter 12, pp. 234-289.
- Johnstone, R., Stet, R.J.M., 1995. The production of gynogenetic Atlantic salmon (*Salmo salar* L.). *Theoretical and Applied Genetics* 90, 819-826.
- Johnstone, R., McLay, H.A., Walsingham, M.V., 1991. Production and performance of triploid Atlantic salmon in Scotland. In: Pepper, V.A. (Ed)., *Proceedings of the Atlantic Canada Workshop on Methods for the Production of Non-maturing Salmonids*, Feb. 19–21, Dartmouth, Nova Scotia. Dept. Fisheries and Oceans, St. Johns, Newfoundland, Canada, pp. 15–36.
- Jonsson, B., Forseth, T., Jensen, A.J., NÆSje, T.F., 2001. Thermal performance of juvenile Atlantic Salmon, *Salmo salar* L. *Functional Ecology* 15, 701-711.
- Julian, R.J., 1998. Rapid growth problems: Ascites and skeletal deformities in broilers. *Poultry Science* 77, 1773-1780.
- Jungalwalla, P., 1991. Production of non-maturing Atlantic salmon in Tasmania. In: Pepper, V.A. (Ed)., *Proceedings of the Atlantic Canada Workshop on Methods for the Production of Non-maturing Salmonids*, Feb. 19–21, Dartmouth, Nova Scotia. Dept. Fisheries and Oceans, St. Johns, Newfoundland, Canada, pp. 47–71.
- Kacem, A., Meunier, F.J., Bagliniere, J.L., 1998. A quantitative study of morphological and histological changes in the skeleton of *Salmo salar* during its anadromous migration. *Journal of Fish Biology* 53, 1096-1109.
- Karsenty, G., 2008. Transcriptional control of skeletogenesis, *Annual Review of Genomics and Human Genetics*, pp. 183-196.
- Karsenty, G., Park, R.-W., 1995. Regulation of Type I Collagen genes expression. *International Reviews of Immunology* 12, 177-185.
- Karsenty, G., Kronenberg, H.M., Settembre, C., 2009. Genetic control of bone formation. *Annual Review of Cell and Developmental Biology* 25, 629-648.
- Kazlauskienė, N., Leliūnaitė, E., Kesminas, V., 2006. Peculiarities of opercular malformations of salmon (*Salmo salar* L.) juveniles reared in the Zeimena Salmon Hatchery. *Acta Zool. Lituanica* 16, 312-316.

- King, H., Lee, P., 1993. Progress report: jaw deformity and respiratory physiology of triploids. Seeking and Solving: Papers from the SALTAS Research and Development Review Seminar. SALTAS, Wayatinah, Tasmania, Australia, pp. 37–44.
- Klemetsen, A., Amundsen, P.A., Dempson, J.B., Jonsson, B., Jonsson, N., O'Connell, M.F., Mortensen, E., 2003. Atlantic salmon *Salmo salar* L., brown trout *Salmo trutta* L. and Arctic charr *Salvelinus alpinus* L.: a review of aspects of their life histories. Ecology of Freshwater Fish 12, 1-59.
- Koumoundouros, G., Maingot, E., Divanach, P., Kentouri, M., 2002. Kyphosis in reared sea bass (*Dicentrarchus labrax* L.): ontogeny and effects on mortality. Aquaculture 209, 49-58.
- Kristensen, T., Haugen, T.O., Rosten, T., Fjellheim, A., Atland, A., Rosseland, B.O., 2012. Effects of production intensity and production strategies in commercial Atlantic salmon smolt (*Salmo salar* L.) production on subsequent performance in the early sea stage. Fish Physiology and Biochemistry 38, 273-282.
- Kuivaniemi, H., Tromp, G., Prockop, D.J., 1997. Mutations in collagens type I, II, III, IX, X and XI cause a spectrum of diseases of bone, cartilage and blood vessels. Hum Mutation 9, 300-315.
- Kvellestad, A., Hoie, S., Thorud, K., Torud, B., Lyngoy, A., 2000. Platyspondyly and shortness of vertebral column in farmed Atlantic salmon *Salmo salar* in Norway - description and interpretation of pathologic changes. Diseases of Aquatic Organisms 39, 97-108.
- Lall, S.P., Lewis-McCrea, L.M., 2007. Role of nutrients in skeletal metabolism and pathology in fish - An overview. Aquaculture 267, 3-19.
- Le Roith, D., Bondy, C., Yakar, S., Liu, J.-L., Butler, A., 2001. The Somatomedin Hypothesis: 2001. Endocrine Reviews 22, 53-74.
- Leclercq, E., Taylor, J.F., Fison, D., Fjelldal, P.G., Diez-Padrisa, M., Hansen, T., Migaud, H., 2011. Comparative seawater performance and deformity prevalence in out-of-season diploid and triploid Atlantic salmon (*Salmo salar*) post-smolts. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 158, 116-125.
- Lee, P., King, H., 1994. Effects of reduced dietary energy on the incidence of jaw deformity in Tasmanian Atlantic salmon. Reports from SALTAS Research and Development Programme. Salmon Enterprises of Tasmania, Wayatinah, Tasmania, pp. 61–69.
- Li, S.-W., Prockop, D.J., Helminen, H., Fassler, R., Lapveteläinen, T., Kiraly, K., Pelttari, A., Arokoski, J., Lui, H., Arita, M., Khillan, J.S., 1995. Transgenic mice with targeted inactivation of the Col2a1 gene for collagen II develop a skeleton with membranous and periosteal bone but no endochondral bone. Genes Dev 9, 2821-2830.
- Li, Y.-J., Kukita, A., Teramachi, J., Nagata, K., Wu, Z., Akamine, A., Kukita, T., 2008. A possible suppressive role of galectin-3 in upregulated osteoclastogenesis accompanying adjuvant-induced arthritis in rats. Lab Invest 89, 26-37.
- Lien, S., Koop, B.F., Sandve, S.R., Miller, J.R., Kent, M.P., 2016. The Atlantic salmon genome provides insights into rediploidization. Nature (London) 533, 200-205.
- Lijalad, M., Powell, M.D., 2009. Effects of lower jaw deformity on swimming performance and recovery from exhaustive exercise in triploid and diploid Atlantic salmon *Salmo salar* L. Aquaculture 290, 145-154.
- Liu, F.-T., Rabinovich, G.A., 2005. Galectins as modulators of tumour progression. Nat Rev Cancer 5, 29-41.
- Liu, W., Selever, J., Murali, D., Sun, X., Brugger, S.M., Ma, L., Schwartz, R.J., Maxson, R., Furuta, Y., Martin, J.F., 2005. Threshold-specific requirements for Bmp4 in mandibular development. Developmental Biology 283, 282-293.
- Lock, E.J., Waagbø, R., Bonga, S.W., Flik, G., 2010. The significance of vitamin D for fish: a review. Aquac. Nutr. 16, 100-116.

- MacLean, A., 1999. Compensatory Growth, Life-history Decisions and Welfare of farmed Atlantic salmon (*Salmo salar* L.) parr. PhD thesis, University of Glasgow.
- Mangos, S., Amaral, A.P., Faul, C., Jüppner, H., Reiser, J., Wolf, M., 2012. Expression of fgf23 and α klotho in developing embryonic tissues and adult kidney of the zebrafish, *Danio rerio*. *Nephrology Dialysis Transplantation* 27, 4314-4322.
- Marschall, E.A., Quinn, T.P., Roff, D.A., Hutchings, J.A., Metcalfe, N.B., Bakke, T.A., Saunders, R.L., Poff, N.L., 1998. A framework for understanding Atlantic salmon (*Salmo salar*) life history. *Canadian Journal of Fisheries and Aquatic Sciences* 55, 48-58.
- Martins, C.I.M., Galhardo, L., Noble, C., Damsgård, B., Spedicato, M.T., Zupa, W., Beauchaud, M., Kulczykowska, E., Massabuau, J.-C., Carter, T., Planellas, S.R., Kristiansen, T., 2011. Behavioural indicators of welfare in farmed fish. *Fish Physiology and Biochemistry* 38, 17-41.
- Maxime, V., 2008. The physiology of triploid fish: current knowledge and comparisons with diploid fish. *Fish and Fisheries* 9, 67-78.
- Mazurais, D., Glynatsi, N., Darias, M.J., Christodouloupoulou, S., Cahu, C.L., Zambonino-Infante, J.-L., Koumoundouros, G., 2009. Optimal levels of dietary vitamin A for reduced deformity incidence during development of European sea bass larvae (*Dicentrarchus labrax*) depend on malformation type. *Aquaculture* 294, 262-270.
- McCarthy, I.D., Carter, C.G., Houlihan, D.F., Johnstone, R., Mitchell, A.I., 1996. The performance of all-female diploid and triploid Atlantic salmon smolts on transfer together to sea water. *Journal of Fish Biology* 48, 545-548.
- McGeachy, S.A., Benfey, T.J., Friars, G.W., 1995. Freshwater performance of triploid Atlantic salmon (*Salmo salar*) in New Brunswick aquaculture. *Aquaculture* 137, 333-341.
- McGeachy, S.A., O'Flynn, F.M., Benfey, T.J., Friars, G.W., 1996. Seawater performance of triploid Atlantic salmon in New Brunswick aquaculture. *Bulletin of the Aquaculture Association of Canada*, 24-28.
- McKay, L.R., Gjerde, B., 1986. Genetic-variation for a spinal deformity in Atlantic salmon, *Salmo salar*. *Aquaculture* 52, 263-272.
- Miller, M., Nichols, P., Barnes, J., Davies, N., Peacock, E., Carter, C., 2006. Regiospecificity profiles of storage and membrane lipids from the gill and muscle tissue of atlantic salmon (*Salmo salar* L.) grown at elevated temperature. *Lipids* 41, 865-876.
- Moren, M., Waagbø, R., Hamre, K., 2011. Chap 4 micronutrients. In: *Larval fish nutrition*, 1st edn. G. J. Holt (Ed.). John Wiley & Sons Inc, West Sussex, UK, pp. 117-149.
- Moss, M.L., 1961. Studies of acellular bone of teleost fish 1. Morphological and systematic variations. *Acta Anatomica* 46, 343-462.
- Murray, L., Bautista, J., James, P., Rimoin, D., 1989. Type II collagen defects in the chondrodysplasias. I. Spondyloepiphyseal dysplasias. *Am J Hum Genet* 45, 5-15.
- Nakabayashi, K., Matsumi, H., Bhalla, A., Bae, J., Mosselman, S., Hsu, S.Y., Hsueh, A.J.W., 2002. Thyrostimulin, a heterodimer of two new human glycoprotein hormone subunits, activates the thyroid-stimulating hormone receptor. *The Journal of Clinical Investigation* 109, 1445-1452.
- O'Flynn, F.M., McGeachy, S.A., Friars, G.W., Benfey, T.J., Bailey, J.K., 1997. Comparisons of cultured triploid and diploid Atlantic salmon (*Salmo salar* L.). *Ices Journal of Marine Science* 54, 1160-1165.
- Ohshima, S., Kuchen, S., Seemayer, C.A., Kyburz, D., Hirt, A., Klinzing, S., Michel, B.A., Gay, R.E., Liu, F.-T., Gay, S., Neidhart, M., 2003. Galectin 3 and its binding protein in rheumatoid arthritis. *Arthritis & Rheumatism* 48, 2788-2795.

- Oppedal, F., Taranger, G.L., Hansen, T., 2003. Growth performance and sexual maturation in diploid and triploid Atlantic salmon (*Salmo salar* L.) in seawater tanks exposed to continuous light or simulated natural photoperiod. *Aquaculture* 215, 145-162.
- Ovenden, J.R., Bywater, R., White, R.W.G., 1993. Mitochondrial DNA nucleotide sequence variation in Atlantic salmon (*Salmo salar*), brown trout (*S. trutta*), rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) from Tasmania, Australia. *Aquaculture* 114, 217-227.
- Parsons, K.J., Albertson, R.C., 2009. Roles for Bmp4 and CaM1 in Shaping the Jaw: Evo-Devo and Beyond, *Annual Review of Genetics*, pp. 369-388.
- Patel, A., Dettleff, P., Hernandez, E., Martinez, V., 2016. A comprehensive transcriptome of early development in yellowtail kingfish (*Seriola lalandi*). *Molecular Ecology Resources* 16, 364-376.
- Pendas, A.M., Balbin, M., Llano, E., Jimenez, M.G., LopezOtin, C., 1997. Analysis and promoter characterization of the human collagenase-3 gene (MMP13). *Genomics* 40, 222-233.
- Piferrer, F., Beaumont, A., Falguiere, J.-C., Flajshans, M., Haffray, P., Colombo, L., 2009. Polyploid fish and shellfish: Production, biology and applications to aquaculture for performance improvement and genetic containment. *Aquaculture* 293, 125-156.
- Powell, M.D., Jones, M.A., Lijalad, M., 2009. Effects of skeletal deformities on swimming performance and recovery from exhaustive exercise in triploid Atlantic salmon. *Diseases of Aquatic Organisms* 85, 59-66.
- Prockop, D.J., Ala-Kokko, L., McLain, D.A., Williams, C., 1997. Can mutated genes cause common osteoarthritis? *Br J Rheumatol* 36, 827-830.
- Quigley, D.T.G., 1995. A lower jaw deformity in juvenile and adult Atlantic salmon (*Salmo salar* L.). *Bulletin of the European Association of Fish Pathologists* 15, 206-209.
- Quillet, E., Gaignon, J.L., 1990. Thermal induction of gynogenesis and triploidy in Atlantic salmon (*Salmo salar*) and their potential interest for aquaculture. *Aquaculture* 89, 351-364.
- Ramirez, F., Rifkin, D.B., 2009. Extracellular microfibrils; contextual platforms for TGF β and BMP signaling. *Current opinion in cell biology* 21, 616-622.
- Rani, P.U., Stringa, E., Dharmavaram, R., Chatterjee, D., Tuan, R.S., Khillan, J.S., 1999. Restoration of normal bone development by human homologue of collagen type II (COL2A1) gene in Col2a1 null mice. *Developmental Dynamics* 214, 26-33.
- Rath, N.C., Huff, G.R., Huff, W.E., Balog, J.M., 2000. Factors Regulating Bone Maturity and Strength in Poultry. *Poultry Science* 79, 1024-1032.
- Richards, A.J., McNinch, A., Martin, H., Oakhill, K., Rai, H., Waller, S., Treacy, B., Whittaker, J., Meredith, S., Poulson, A., Snead, M.P., 2010. Stickler syndrome and the vitreous phenotype: mutations in COL2A1 and COL11A1. *Human Mutation* 31, E1461-E1471.
- Roberts, R.J., Hardy, R.W., Sugiura, S.H., 2001. Screamer disease in Atlantic salmon, *Salmo salar* L., in Chile. *Journal of Fish Diseases* 24, 543-549.
- Robinson, P.N., Godfrey, M., 2000. The molecular genetics of Marfan syndrome and related microfibrilopathies. *Journal of Medical Genetics* 37, 9-25.
- Rojas, H., Maretto, D., Cassigoly, J., Uribe, C., Campisto, J., Urcelay, S., 2000. A case control study of jaw deformity in *Salmo salar* in Chile.
- Rossert, J., Terraz, C., Dupont, S., 2000. Regulation of type I collagen genes expression. *Nephrology Dialysis Transplantation* 15, 66-68.
- Rowe, P.S.N., 2012. Regulation of Bone–Renal Mineral and Energy Metabolism: The PHEX, FGF23, DMP1, MEPE ASARM Pathway. *Critical Reviews in Eukaryotic Gene Expression* 22, 61-86.

- Sacobie, C.F.D., Glebe, B.D., Barbeau, M.A., Lall, S.P., Benfey, T.J., 2012. Effect of strain and ploidy on growth performance of Atlantic salmon, *Salmo salar*, following seawater transfer. *Aquaculture* 334, 58-64.
- Sadler, J., 2000. Comparison of aspects of the physiology and morphology of diploid and triploid Atlantic salmon *Salmo Salar*. PhD thesis University of Tasmania.
- Sadler, J., Pankhurst, P.M., King, H.R., 2001. High prevalence of skeletal deformity and reduced gill surface area in triploid Atlantic salmon (*Salmo salar* L.). *Aquaculture* 198, 369-386.
- Sadler, J., Wells, R.M.G., Pankhurst, P.M., Pankhurst, N.W., 2000a. Blood oxygen transport, rheology and haematological responses to confinement stress in diploid and triploid Atlantic salmon, *Salmo salar*. *Aquaculture* 184, 349-361.
- Sadler, J., Pankhurst, N.W., Pankhurst, P.M., King, H., 2000b. Physiological stress responses to confinement in diploid and triploid Atlantic salmon. *Journal of Fish Biology* 56, 506-518.
- Sahlman, J., Pitkänen, M.T., Prockop, D.J., Arita, M., Li, S.-W., Helminen, H.J., Långsjö, T.K., Puustjärvi, K., Lammi, M.J., 2004. A human COL2A1 gene with an Arg519Cys mutation causes osteochondrodysplasia in transgenic mice. *Arthritis & Rheumatism* 50, 3153-3160.
- Savontaus, M., Rintala-Jämsä, M., Morko, J., Rönning, O., Metsäranta, M., Vuorio, E., 2004. Abnormal craniofacial development and expression patterns of extracellular matrix components in transgenic D α 1 mice harboring a deletion mutation in the type II collagen gene. *Orthodontics & Craniofacial Research* 7, 216-226.
- Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* 9, 671-675.
- Sire, J.-Y., Huyseune, A., Meunier, F.J., 1990. Osteoclasts in teleost fish: Light and electron-microscopical observations. *Cell and Tissue Research* 260, 85-94.
- Smedley, M.A., Clokie, B.G.J., Migaud, H., Campbell, P., Walton, J., Hunter, D., Corrigan, D., Taylor, J.F., 2016. Dietary phosphorous and protein supplementation enhances seawater growth and reduces severity of vertebral malformation in triploid Atlantic salmon (*Salmo salar* L.). *Aquaculture* 451, 357-368.
- Solbakken, V.A., Hansen, T., Stefansson, S.O., 1994. Effects of photoperiod and temperature on growth and parr-smolt transformation in Atlantic salmon (*Salmo salar* L.) and subsequent performance in seawater. *Aquaculture* 121, 13-27.
- Spoorendonk, K.M., Hammond, C.L., Huitema, L.F.A., Vanoevelen, J., Schulte-Merker, S., 2010. Zebrafish as a unique model system in bone research: the power of genetics and imaging. *Journal of Applied Ichthyology* 26, 219-224.
- Stephan, M., Hobsbawn, P., 2015. Australian fisheries and aquaculture statistics 2014, Fisheries Research and Development Corporation project 2014/245. ABARES, Canberra, November. CC BY 3.0.
- Stock, M., Schäfer, H., Stricker, S., Gross, G., Mundlos, S., Otto, F., 2003. Expression of Galectin-3 in skeletal tissues is controlled by Runx2. *Journal of Biological Chemistry* 278, 17360-17367.
- Stockwell, R.A., 1979. Biology of cartilage cells. Cambridge: Cambridge University Press.
- Suazo, J., Tapia, J.C., Santos, J.L., Castro, V.G., Colombo, A., Blanco, R., 2011. Risk variants in BMP4 promoters for nonsyndromic cleft lip/palate in a Chilean population. *BMC Medical Genetics* 12, 1-9.
- Sullivan, M., Hammond, G., Roberts, R.J., Manchester, N.J., 2007a. Spinal deformation in commercially cultured Atlantic salmon, *Salmo salar* L.: a clinical and radiological study. *Journal of Fish Diseases* 30, 745-752.

- Sullivan, M., Reid, S.W.J., Ternent, H., Manchester, N.J., Roberts, R.J., Stone, D.A.J., Hardy, R.W., 2007b. The aetiology of spinal deformity in Atlantic salmon, *Salmo salar* L.: influence of different commercial diets on the incidence and severity of the preclinical condition in salmon parr under two contrasting husbandry regimes. *Journal of Fish Diseases* 30, 759-767.
- Sutterlin, A.M., Collier, C., 1991. Some observation on the commercial use of triploid rainbow trout and Atlantic salmon in Newfoundland, Canada. *Can. Tech. Rep. Fish. Aquat. Sci.* 1789, 89-96.
- Sutterlin, A.M., Holder, J., Benfey, T.J., 1987. Early survival rates and subsequent morphological abnormalities in landlocked, anadromous and hybrid (landlocked x anadromous) diploid and triploid Atlantic salmon. *Aquaculture* 64, 157-164.
- Tahimic, C.G.T., Wang, Y., Bikle, D.D., 2013. Anabolic effects of IGF-1 signaling on the skeleton. *Frontiers in Endocrinology* 4, 6.
- Takle, H., Baeverfjord, G., Lunde, M., Kolstad, K., Andersen, O., 2005. The effect of heat and cold exposure on HSP70 expression and development of deformities during embryogenesis of Atlantic salmon (*Salmo salar*). *Aquaculture* 249, 515-524.
- Tang, S.Y., Herber, R.P., Ho, S.P., Alliston, T., 2012. Matrix Metalloproteinase-13 is required for osteocytic perilacunar remodeling and maintains bone fracture resistance. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 27, 1936-1950.
- Tanikawa, R., Tanikawa, T., Hirashima, M., Yamauchi, A., Tanaka, Y., 2010. Galectin-9 induces osteoblast differentiation through the CD44/Smad signaling pathway. *Biochemical and Biophysical Research Communications* 394, 317-322.
- Taranger, G.L., Carrillo, M., Schulz, R.W., Fontaine, P., Zanuy, S., Felip, A., Weltzien, F.-A., Dufour, S., Karlsen, Ø., Norberg, B., Andersson, E., Hansen, T., 2010. Control of puberty in farmed fish. *General and Comparative Endocrinology* 165, 483-515.
- Taylor, J.F., Preston, A.C., Guy, D., Migaud, H., 2011. Ploidy effects on hatchery survival, deformities, and performance in Atlantic salmon (*Salmo salar*). *Aquaculture* 315, 61-68.
- Taylor, J.F., Leclercq, E., Preston, A.C., Guy, D., Migaud, H., 2012. Parr-smolt transformation in out-of-season triploid Atlantic salmon (*Salmo salar* L.). *Aquaculture* 362, 255-263.
- Taylor, J.F., Bozzolla, P., Frenzl, B., Matthew, C., Hunter, D., Migaud, H., 2014. Triploid Atlantic salmon growth is negatively affected by communal ploidy rearing during seawater grow-out in tanks. *Aquaculture* 432, 163-174.
- Taylor, J.F., Sambras, F., Mota-Velasco, J., Guy, D.R., Hamilton, A., Hunter, D., Corrigan, D., Migaud, H., 2013. Ploidy and family effects on Atlantic salmon (*Salmo salar*) growth, deformity and harvest quality during a full commercial production cycle. *Aquaculture* 410-411, 41-50.
- Taylor, J.F., Waagbø, R., Diez-Padrisa, M., Campbell, P., Walton, J., Hunter, D., Matthew, C., Migaud, H., 2015. Adult triploid Atlantic salmon (*Salmo salar*) have higher dietary histidine requirements to prevent cataract development in seawater. *Aquaculture Nutrition* 21, 18-32.
- Tiller, G., Rimoin, D., Murray, L., Cohn, D., 1990. Tandem duplication within a type II collagen gene (COL2A1) exon in an individual with spondyloepiphyseal dysplasia. *Proc Natl Acad Sci USA* 87, 3889-3893.
- Upholt, W.B., 1989. In: Olsen BR, Ninmi ME, editors. *Collagen, Vol V*. Boca Raton FL: CRC Press. 31-49.
- Vagsholm, I., Djupvik, H.O., 1998. Risk factors for spinal deformities in Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases* 21, 47-53.

- Venegas, F., Montiel, E., Forno, P., Rojas, M., 2003. Histology of the jaw deformation in salmon of southern Chile. *International Journal of Morphology* 21, 211-219.
- Ventura, T., Manor, R., Aflalo, E.D., Weil, S., Khalaila, I., Rosen, O., Sagi, A., 2011. Expression of an androgenic gland-specific insulin-like peptide during the course of prawn sexual and morphotypic differentiation. *ISRN Endocrinology* 2011, 11.
- Vieira, F.A., Thorne, M.A.S., Stueber, K., Darias, M., Reinhardt, R., Clark, M.S., Gisbert, E., Power, D.M., 2013. Comparative analysis of a teleost skeleton transcriptome provides insight into its regulation. *General and Comparative Endocrinology* 191, 45-58.
- Vikkula, M., Metsaranta, M., Ala-Kokko, L., 1994. Type II collagen mutations in rare and common cartilage diseases. *Ann Med* 26, 107-114.
- Vinik, Y., Shatz-Azoulay, H., Vivanti, A., Hever, N., Levy, Y., Karmona, R., Brumfeld, V., Baraghithy, S., Attar-Lamdar, M., Boura-Halfon, S., Bab, I., Zick, Y., 2015. The mammalian lectin galectin-8 induces RANKL expression, osteoclastogenesis, and bone mass reduction in mice. *eLife* 4, e05914.
- Waagbø, R., 2010. Water soluble vitamins in fish ontogeny. *Aqua. Res.* 41, 733-744.
- Waldenstedt, L., 2006. Nutritional factors of importance for optimal leg health in broilers: A review. *Animal Feed Science and Technology* 126, 291-307.
- Wang, R.N., Green, J., Wang, Z., Deng, Y., Qiao, M., Peabody, M., Zhang, Q., Ye, J., Yan, Z., Denduluri, S., Idowu, O., Li, M., Shen, C., Hu, A., Haydon, R.C., Kang, R., Mok, J., Lee, M.J., Luu, H.L., Shi, L.L., 2014. Bone Morphogenetic Protein (BMP) signaling in development and human diseases. *Genes & Diseases* 1, 87-105.
- Wargelius, A., Fjelldal, P.G., Nordgarden, U., Hansen, T., 2009. Continuous light affects mineralization and delays osteoid incorporation in vertebral bone of Atlantic salmon (*Salmo salar* L.). *Journal of Experimental Biology* 212, 656-661.
- Wargelius, A., Fjelldal, P.G., Benedet, S., Hansen, T., Bjornsson, B.T., Nordgarden, U., 2005. A peak in gh-receptor expression is associated with growth activation in Atlantic salmon vertebrae, while upregulation of igf-I receptor expression is related to increased bone density. *General and Comparative Endocrinology* 142, 163-168.
- Whyte, M.P., 1994. Hypophosphatasia and the role of alkaline phosphatase in skeletal mineralization. *Endocrine Reviews* 15, 439-461.
- Witten, P.E., Hall, B.K., 2002. Differentiation and growth of kype skeletal tissues in anadromous male Atlantic Salmon (*Salmo salar*). *International Journal of Developmental Biology* 46, 719-730.
- Witten, P.E., Hall, B.K., 2003. Seasonal changes in the lower jaw skeleton in male Atlantic salmon (*Salmo salar* L.): remodelling and regression of the kype after spawning. *Journal of Anatomy* 203, 435-450.
- Witten, P.E., Huysseune, A., 2009. A comparative view on mechanisms and functions of skeletal remodelling in teleost fish, with special emphasis on osteoclasts and their function. *Biological Reviews* 84, 315-346.
- Witten, P.E., Hall, B.K., 2015. Teleost Skeletal Plasticity: Modulation, Adaptation, and Remodelling. *Copeia* 103, 727-739.
- Witten, P.E., Huysseune, A., Hall, B.K., 2010. A practical approach for the identification of the many cartilaginous tissues in teleost fish. *Journal of Applied Ichthyology* 26, 257-262.
- Witten, P.E., Obach, A., Huysseune, A., Baeverfjord, G., 2006. Vertebrae fusion in Atlantic salmon (*Salmo salar*): Development, aggravation and pathways of containment. *Aquaculture* 258, 164-172.
- Witten, P.E., Gil-Martens, L., Hall, B.K., Huysseune, A., Obach, A., 2005. Compressed vertebrae in Atlantic salmon *Salmo salar*: evidence for metaplastic chondrogenesis as a skeletogenic response late in ontogeny. *Diseases of Aquatic Organisms* 64, 237-246.

- Witten, P.E., Gil-Martens, L., Huysseune, A., Takle, H., Hjelde, K., 2009. Towards a classification and an understanding of developmental relationships of vertebral body malformations in Atlantic salmon (*Salmo salar* L.). *Aquaculture* 295, 6-14.
- Witten, P.E., Huysseune, A., Franz-Odegaard, T., Fedak, T., Vickaryous, M., Cole, A., Hall, B.K., 2004. Acellular teleost bone: primitive or derived, dead or alive? *The Palaeontology Newsletter* 55, 37-41.
- Xue, F., Rabie, A.B.M., Luo, G., 2014. Analysis of the association of COL2A1 and IGF-1 with mandibular prognathism in a Chinese population. *Orthodontics & Craniofacial Research* 17, 144-149.
- Yakar, S., Rosen, C.J., Beamer, W.G., Ackert-Bicknell, C.L., Wu, Y., Liu, J.-L., Ooi, G.T., Setser, J., Frystyk, J., Boisclair, Y.R., LeRoith, D., 2002. Circulating levels of IGF-1 directly regulate bone growth and density. *The Journal of Clinical Investigation* 110, 771-781.
- Ytteborg, E., Torgersen, J., Baeverfjord, G., Takle, H., 2010a. Morphological and molecular characterization of developing vertebral fusions using a teleost model. *BMC Physiology* 10, 13-13.
- Ytteborg, E., Torgersen, J., Baeverfjord, G., Takle, H., 2012. The Atlantic Salmon (*Salmo salar*) Vertebra and Cellular Pathways to Vertebral Deformities in: *Health and Environment in Aquaculture*, Edmir, D.C. (Ed.), Available from: <http://www.intechopen.com/books/health-and-environment-in-aquaculture/the-atlantic-salmon-salmo-salar-vertebra-and-cellular-pathways-to-vertebral-deformities>.
- Ytteborg, E., Baeverfjord, G., Torgersen, J., Hjelde, K., Takle, H., 2010b. Molecular pathology of vertebral deformities in hyperthermic Atlantic salmon (*Salmo salar*). *BMC Physiology* 10, 16.
- Ytteborg, E., Vegusdal, A., Witten, P.E., Berge, G.M., Takle, H., Ostbye, T.K., Ruyter, B., 2010c. Atlantic salmon (*Salmo salar*) muscle precursor cells differentiate into osteoblasts in vitro: polyunsaturated fatty acids and hyperthermia influence gene expression and differentiation. *Biochim. Biophys. Acta-Mol. Cell Biol. Lip.* 1801, 127-137.
- Zapata, A., Diez, B., Cejalvo, T., Frias, C.G., Cortes, A., 2006. Ontogeny of the immune system of fish. *Fish & shellfish immunology* 20, 126-136.